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# Dependence of Allosteric Modulation of Glycine Receptor Function on Agonist Efficacy

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# Dependence of Allosteric Modulation of Glycine Receptor Function on Agonist Efficacy

**by**

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# Dedication

This dissertation is dedicated to my family, blood and otherwise. To my mother, Jeannie, for always believing in me. To my brother, Claus, who dropped everything to be here during the worst of it. To Leo, for reminding me how important the simple things can be. To Wolfgang, who inspired me with his ambition and discipline. To the unstoppable Sarah Burke, my greatest role model. Most of all, to my husband, Seann, who lent me his strength whenever I ran out. I couldn't have done it without you.

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# Dependence of Allosteric Modulation of Glycine Receptor Function on Agonist Efficacy

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The glycine receptor (GlyR) is a ligand-gated chloride channel that mediates inhibitory neurotransmission in the brain and spinal cord. Numerous allosteric modulators act on the GlyR including divalent cations, such as zinc, as well as drugs of abuse including ethanol, inhalants, anesthetics and cannabinoids. GlyRs mediate some of the rewarding effects of addictive drugs and modulate drug related behaviors through activity in the mesolimbic dopamine reward pathway. GlyR activity, however, can differ depending on whether the receptor is activated by the high-efficacy agonist, glycine, or taurine which has much lower efficacy at wild-type GlyRs. As glycine and taurine are believed to activate the GlyR *in vivo*, it is crucial that we understand receptor function and allosteric modulation of receptors in response to both agonists. We used two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes to study the effects of zinc and ethanol on wildtype glycine receptors activated by glycine or taurine. We determined that the magnitude of allosteric modulation was higher, overall, at taurine-

gated receptors and hypothesized that this may be related to the difference in efficacies. Considering that GlyR mutants can affect agonist sensitivity and the response to allosteric modulators, we wondered whether changes in allosteric modulation at mutant receptors could be due to changes in agonist efficacy caused by these mutations. We tested this hypothesis by characterizing ethanol and zinc modulation of taurine currents at GlyR mutants that showed an increase ($\alpha1^{W170S}$) or decrease ($\alpha1^{A52S}$) in taurine efficacy. We found that the W170S mutation increases the relative efficacy of taurine to a level that is comparable with glycine and abolishes ethanol enhancement of maximally-effective taurine currents. We determined that the difference in ethanol potentiation of low taurine currents between W170S and WT receptors is due to zinc enhancement of WT currents. Ethanol modulation of these receptors was equal in the presence of tricine. This suggests that ethanol also increases taurine affinity at W170S. A52S, on the other hand, displayed reduced taurine efficacy and increased ethanol modulation. This work provides evidence of a mechanism by which the degree of allosteric modulation of glycine receptor function is dependent on agonist efficacy.

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# List of Abbreviations

| | |
|---|---|
| 2,6-DTBP | 2,6-di-ert-butylphenol |
| 2-AG | 2-arachidonylglycerol |
| 5-$HT_3R$ | serotonin type 3 receptor |
| $\Delta^9$-THC | delta-9-tetrahydrocannabinol |
| Å | angstrom |
| AEA | anandamide |
| ANOVA | analysis of variance |
| AUD | alcohol use disorder |
| CBD | cannabidiol |
| CLR | cys-loop receptor |
| CSF | cerebrospinal fluid |
| CNS | central nervous system |
| CTB | cyanotriphenylborate |
| DH-CBD | dihydroxyl-cannabidiol |
| EC | effective concentration |
| ECD | extracellular domain |
| EtOH | ethanol |
| $GABA_AR$ | γ-aminobutyric acid type A receptor |
| Gly | glycine |
| GlyR | Glycine Receptor |
| HEK | human embryonic kidney |
| IL | intracellular loop |
| LGIC | ligand gated ion channel |

| | |
|---|---|
| LORR | loss of righting reflex |
| MBS | modified Barth's saline |
| nAc | nucleus accumbens |
| nAChR | nicotinic acetylcholine receptor |
| PFC | prefrontal cortex |
| $PGE_2$ | prostaglandin $E_2$ |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PTX | picrotoxin |
| SEM | standard error mean |
| Tau | taurine |
| TCE | 1,1,1-trichloroethane |
| TCY | trichloroethylene |
| TM | transmembrane |
| Tri | tricine |
| VTA | ventral tegmental area |
| WT | wildtype |

## Amino Acid Residue Abbreviations and Naming Conventions

The amino acid residues mentioned in this manuscript will often be abbreviated using the single letter codes listed below. Point mutations will be described using the following convention: original residue – residue position – new residue. For example, the mutation of an alanine residue to serine at position 52 would be referred to as A52S.

**Single-letter amino acid abbreviations**

| | | | |
|---|---|---|---|
| A | Alanine | M | Methionine |
| C | Cysteine | N | Asparagine |
| D | Aspartate | P | Proline |
| E | Glutamate | Q | Glutamine |
| F | Phenylalanine | R | Arginine |
| G | Glycine | S | Serine |
| H | Histidine | T | Threonine |
| I | Isoleucine | V | Valine |
| K | Lysine | W | Tryptophan |
| L | Leucine | Y | Tyrosine |

# Chapter 1: Introduction

## 1.1 - Cys-Loop Family of Ligand-Gated Ion Channels

The nervous system encompasses a complex network of specialized cells responsible for transmitting information throughout the body using a combination of chemical and electrical signals. Ligand-gated ion channels (LGICs) receive chemicals signals in the form of specific ligands that bind to the channel, regulating rapid ion flux across the cell membrane. This, in turn, modulates bioelectrical communication by generating changes in membrane potential on the order of milliseconds (Langosch et al., 1990). Cys-loop receptors (CLRs) form a family of ligand-gated ion channels (LGICs) that share a similar structure (Figure 1.1). Cation-conducting CLRs include nicotinic acetylcholine receptors (nACh), serotonin type three receptors (5-$HT_3$), and glutamate-gated chloride channels (GluCl) receptors. Additional members conduct anions such as GABA receptors ($GABA_AR$, $GABA_CR$), glycine receptors (GlyR), modulation of locomotor defective channels (MOD-1) and excitatory GABA-gated cation channels (EXP-1) (Lester et al., 2004; Pless et al., 2008).

All CLRs receptors are homo- or heteromeric pentamers with subunits arranged radially around an ion conducting pore. Each subunit contains of a large extracellular domain (ECD) composed primarily of beta sheets and contains the signature cys-loop, a closed loop formed by a disulfide bond between cysteine residues (Sine and Engle, 2006). The extracellular domain (ECD) contains binding sites for agonists, antagonists and various modulators. Glycine receptors contain an additional cys-loop that contributes

to the glycine binding domain (Webb and Lynch, 2007). Homologous receptors have also been identified in prokaryotes. ELIC and GLIC receptors, found in *Erwinia chrysanthemi* and *Gloeobacter violaceus*, respectively, share a similar structure with CLRs but lack the characteristic cys-loop found in eukaryotic channels (daCosta and Baenziger, 2013; Lester et al., 2004; Miller and Smart, 2010; Nys et al., 2013).

The membrane-spanning region of each subunit is composed of four α-helices (TM1-4) arranged clockwise with the second transmembrane helix (TM2) from each subunit lining a central pore and the fourth transmembrane helix facing the plasma membrane. There is also an intracellular domain in each subunit connecting TM3 and TM4 which varies considerably in size between CLRs and is involved in modulation of receptor function (Burgos et al., 2015; Sine and Engle, 2006; Webb and Lynch, 2007).

A great deal of structural information has been gleaned from the crystal structure of the Acetylcholine Binding Protein (AChBP) from *Lymnea stagnalis* which shares 20-24% amino acid sequence homology with nAChR (Colquhoun and Sivilotti, 2004; Webb and Lynch, 2007). Like CLRs, the AChBP is pentameric with agonist binding sites located at subunit interfaces. Not all subunits contribute to agonist binding and, due to variations in subunit composition, not all intersubunit interfaces and binding sites on a given receptor are identical (Miller and Smart, 2010; Shan et al., 2003). The nAChR is composed of two alpha and three non-alpha subunits and has two agonist binding sites whereas homomeric CLRs contain five identical binding sites (Colquhoun and Sivilotti, 2004).

A cation-π interaction between each bound agonist and an aromatic residue within the binding pocket is highly conserved among CLRs, although the exact location of the critical aromatic amino acid varies among members. In nACh and 5-$HT_3$ receptors a tryptophan residue in Loop B forms this critical interaction while a tyrosine in Loop A performs the same function in $GABA_A$Rs. In GlyRs, this bond involves a phenylalanine in Loop B (Miller and Smart, 2010; Pless et al., 2008). Additional aromatic amino acids in the ligand binding site contribute to ligand recognition (Nys et al., 2013).

All CLRs require the presence of at least two bound agonist molecules for full activation. After ligand binding, the signal is conveyed to the channel within microseconds across a distance of ~50-60 Å (Miller and Smart, 2010; Sine and Engle, 2006). Receptor activation is believed to involve a series of structural changes described as a 'conformational wave' that originates at the binding site and propagates across the receptor (Grossman et al., 2000). This idea was further supported by rate-equilibrium free energy relationship (REFER) experiments that modeled the gating process as a series of domain shifts (Sine and Engle, 2006).

Ligand binding is immediately followed by the movement of Loop C, closing the binding pocket. Movement of Loop A, which normally helps stabilize the receptor in the closed state, also occurs early in the activation process (Miller and Smart, 2010). Conveying this information to the pore requires interactions between the ECD and transmembrane domains that are crucial for channel gating. Interactions between residues in the LBD between β strands 1 and 2, 8 and 9, as well as the cys-loop, and the

M2-M3 linker have all been shown to contribute to the gating process (Sine and Engle, 2006).

The channel forming TM2 helices from each subunit are bent at the midpoint in the 9' to 13' region, with the extracellular halves of each tilted away from each other such that the center of the pore is more constricted than the outer regions (Webb and Lynch, 2007). The ion conduction pathway consists of 7 rings of amino acids from the M2 helices that are oriented into the lumen of the pore (Nys et al., 2013). Hydrophobic interactions among a conserved ring of leucine residues at the 9' position form part of the principal gate that restricts ion movement through the channel when in the closed state (Labarca et al., 1995; Miller and Smart, 2010). Rings of charged residues at either end of the channel help determine ion selectivity while charged residues at the -1' position further assist in charge discrimination. The ECD may also contribute to rapid conductance by helping to concentrate ions in the vestibule. (Miller and Smart, 2010; Sine and Engle, 2006)

Based on the cryo-EM structure of the nAChR in the open state, we know that pore opening involves a clockwise rotation and outward tilt of each M2 region toward the M1 and M3 helices that widens the constriction point from 3 Å in the closed state to ~8 Å in the open state (daCosta and Baenziger, 2013; Miller and Smart, 2010; Sine and Engle, 2006). This flexibility of the M2 helices is partially dependent on highly conserved glycine residues in the M1-M2 and M2-M3 loops (Miller and Smart, 2010).

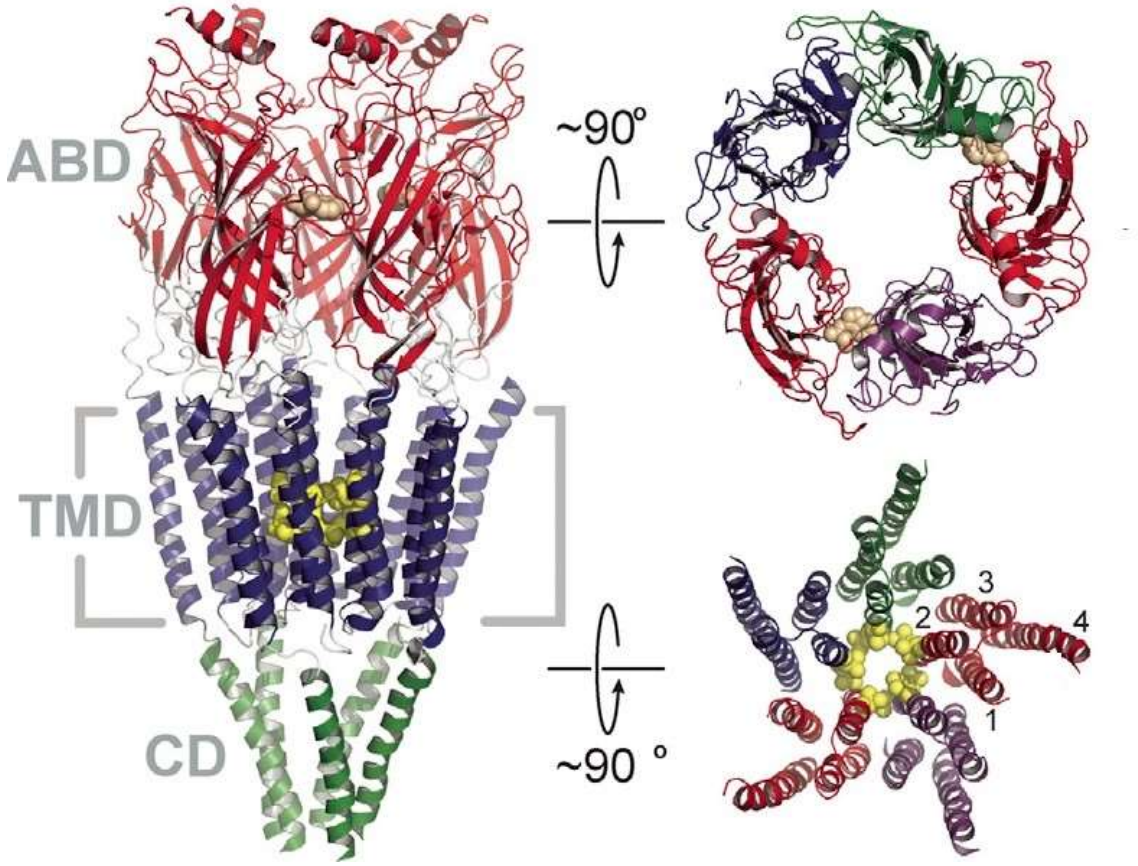


**Figure 1.1: Basic Structure of a Cys-loop Receptor**

Left: Structure of the nicotinic acetylcholine receptor (nAChR) from Torpedo (Protein Data Bank [PDB] ID code 2BG9. Side view of the extracellular agonist binding domain (red), the transmembrane region containing the pore (blue) and the intracellular cytoplasmic domain (green). Tan spheres in the ECD correspond to residues that form the ligand binding site and yellow spheres in the transmembrane domain represent R groups that form the channel gate.
Top Right: nAChR viewed from outside the cell showing the extracellular domain. Each subunit is shown in a different color.
Bottom Right: Top view of the transmembrane domain showing the clockwise arrangement of the TM1-4 helices for each subunit.
Adapted from *Da Costa, C.J.B., Baenziger, J.E., 2013. Gating of pentameric ligand-gated ion channels: structural insights and ambiguities. Structure. 21, 1271-1283.*

## 1.2 - The Glycine Receptor

### 1.2.1 - Basic Structure

The Glycine receptor (GlyR) is responsible for inhibitory neurotransmission in the brain and spinal cord. GlyR α and β subunits were first isolated from mammalian spinal cord via affinity purification (Betz, 1987). Four α subunits have been identified (α1-α4) that share 80-90% sequence homology (Betz and Laube, 2006) and are believed to have developed as a result of gene duplication events. A single β subunit has been identified which shares approximately 47% sequence identity with the α1 subunit and serves to anchor heteromeric receptors to the cytoskeleton of post-synaptic neurons through its interaction with the protein gephyrin (Lynch, 2004). α1, α2, α3 and β form functional receptors in humans. The α4 subunit, although found in mice, chicks and zebrafish, is only a pseudogene in humans (Bowery and Smart 2006; Lynch 2009, 2004).

GlyRs occur as homomeric receptors composed of a single type of α subunit or as αβ heteromers. Kuhse et al. (1993) constructed chimeric receptors of α and β subunits in order to identify regions of the receptor involved in subunit assembly. They discovered a portion of the ECD in the β subunit that is found at subunit interfaces and is required for the invariant stoichiometric assembly of heteromeric GlyR. They reported that functional heteromeric GlyRs are composed of 3α and 2β subunits. However, the exact stoichiometery of heteromeric GlyRs is still debated and has been reported as both 3α:2β

(Becker et al., 1988, Betz et al., 1993, Kuhse et al., 1993; Burzomato et al., 2003) and 2α:3β (Grudzinska et al., 2005).

The extracellular domain of the GlyR forms a barrel-like structure consisting of 10 β-strands and two α-helices at the N-terminus between the β3 and β4 strands. Agonist binding sites are located between adjacent subunits and are formed by loops A/B/C of the principal (+) face and involve a number of residues including: F44, F63, R65, L117, L127, and S129 in the α1 subunit. The complementary face of the LBD is formed by β-strands D/E/F and involves F159, Y202, T204 and F207 (Burgos et al., 2016). In GlyRs containing αl subunits, Phe159 forms a crucial cation-π bond with the amine nitrogen of the agonist (Miller and Smart, 2010; Pless et al., 2008). Based on the model proposed by Colquhoun and Sivilotti (2004) GlyRs require a minimum of 3 bound agonists for full activation.

### 1.2.2 - Distribution and Function

GlyR subunits are unevenly distributed in the Central Nervous System (CNS). Early *in situ* hybridization experiments in rats showed αl transcripts in the spinal cord, brain stem, thalamus, hypothalamus and superior and inferior colliculi. α2 mRNAs were identified in the cerebral cortex, hippocampus, thalamus, hypothalamus, geniculate nuclei, and, to a lesser extent, in the brain stem and spinal cord, while α3 was found in the cerebellum, olfactory bulb, hippocampus, hypothalamus, mesencephalon and spinal cord, particularly in the dorsal horn. Distribution of transcripts for the β subunit showed

widespread distribution throughout the brain and spinal cord (Betz et al., 1993; Malosia et al., 1991a).

Baer et al., 2009 used immunohistochemistry in conjunction with light and confocal laser scanning microscopy to probe the cellular and subcellular localization of GlyRs in the human forebrain, brainstem and spinal cord. This study demonstrated a similar GlyR distribution to that previously described in immunohistochemical studies of the rat brainstem and spinal cord. GlyRs have also been identified in the nucleus accumbens and prefrontal cortex (Baer et al. 2009; Jonsson et al. 2012a, 2009b; Lynch 2004). The β subunit is expressed throughout the retina while α subunits, which are also present at high levels, have distinct patterns of distribution. α1 mRNA has been found in bipolar and some ganglion cells while α2 subunits are present in amacrine cells of the inner nuclear layer and the ganglion cell layer. α3 are distributed throughout the entire inner nuclear layer and, to a lower degree in the ganglion cell layer (Betz and Laube, 2006). Glycine receptors also perform important functions outside of the CNS. Extrasynaptic GlyR are involved in the acrosomal reaction that fuses sperm and egg and have been found in macrophages and leucocytes where they help modulate the inflammatory immune response (Webb and Lynch, 2007).

Expression of the sodium-potassium-chloride cotransporter ($NKCC_1$) is high in the embryonic neurons, resulting in the accumulation of chloride inside the cell. Activation of α2-containing GlyRs on these cell results in outward flux of $Cl^-$ which causes depolarization. This GlyR-mediated excitation is believed to be crucial for

neuronal differentiation and synaptogenesis during early development; however, due to the low concentration of glycine in the neocortex, it is likely that GlyR in this region are being activated by a different agonist. Taurine is present at high concentrations throughout the brain and taurine deprivation is known to lead to defects in cortical development, making it a likely candidate (Flint et al., 1998; Webb and Lynch, 2007). In later stages of development $NKCC_1$ expression declines while expression of the $K+/Cl^-$ cotransporter ($KCC_2$) increases, causing a reduction in intracellular chloride concentrations, eventually reaching concentrations of 3-5 mM in adult neurons. Due to this shift in the chloride gradient, activation of GlyRs in mature neurons produce an influx of $Cl^-$ which hyperpolarizes the cell and, as a result, causes inhibition (Aguayo et al., 2004; Webb and Lynch, 2007).

Despite their high sequence homology, the various subunits that make up glycine receptors differ in their sensitivities and responses to different agonists, antagonists and modulators. Differential expression of GlyR subtypes in different brain regions further adds to their functional diversity *in vivo*. The expression and distribution of GlyR subunits is also known to change during development.

In the spinal cord, α2 homomers present in early development are largely replaced by α1β heteromers in adults. α1 expression is low in early development and increases rapidly after birth (Webb and Lynch, 2007). There are two isoforms of α1 with similar distribution patterns (Lynch, 2004). The human WT α1 isoform 1 contains an 8-amino acid insertion in the intracellular loop between TM3 and TM4 that includes a potential

protein kinase phosphorylation consensus sequence. The same insertion can be found in the rat $\alpha 1^{ins}$ variant (Lynch, 2004; Malosia et al., 1991a, 1991b).

GlyRs composed of α2 subunits predominate during embryonic development but their expression declines rapidly after birth (Webb and Lynch, 2007). In the adult forebrain, however, the α2 subunit persists as a heteromer with the β subunit (Jonsson et al., 2012). Homomeric α2 receptors are also maintained at high levels in the adult retina and auditory brain stem (Lynch, 2004). In adults, α2 homomeric receptors are mainly found in extrasynaptic locations. α2 homomeric GlyRs expressed heterologously in *Xenopus laevis* oocytes show a decreased response to the partial agonists taurine and β-alanine relative to glycine, as well as reduced sensitivity to the antagonist strychnine compared to α1 (Schmieden et al., 1992).

In rats, the α2 subunit also has two spice variants, α2A and α2B, that differ by two residues at positions 58 and 59 in the NTD. The α2B variant possesses a valine at position 58 and a threonine at position 59 while the α2A variant contains isoleucine and alanine, respectively (Miller et al., 2004). In embryonic tissues, the α2A-isoform predominates whereas α2B is more highly expressed in adults (Lynch, 2004). Glycine, β-alanine and taurine $EC_{50}$ values are left-shifted in α2B relative to α2A. Furthermore, currents produced by maximally effective concentrations of taurine at α2B are comparable to those produced by saturating concentrations of glycine. In α2A, maximal taurine currents are lower than glycine currents indicating that taurine has lower efficacy

at this isoform. Both isoforms exhibit similar sensitivity to strychnine and zinc but α2A is ~5X more sensitive to picrotoxin (PTX) (Miller et al., 2004). An additional variant of the α2 subunit, α2*, differs from wildtype by a single point mutation G167E that renders it insensitive to strychnine (Lynch, 2004).

Expression of the α3 subunit increases during development but is only detected postnatally (Malosia et al., 1991a; Burgos et al., 2016). The expression pattern of α3 mirrors that of α1 although at lower levels outside of the dorsal horn (Webb and Lynch, 2007). In the hippocampus, α3 homomeric receptors are mainly extrasynaptic where they are believed to play a role in tonic inhibition along with α2 homomeric GlyRs (Langlhofer and Villmann, 2016). The human α3 subunit has two spice variants, α3K (short) and α3L (long), that are similarly distributed (Lynch, 2004). α3L, as well as the rat α3 subunit, contains a 15-amino acid splice cassette in the intracellular TM3-4 loop that may provide up to three phosphorylation consensus sites at T358, Y367 and S370. This insertion has a marked effect on receptor desensitization. α3K exhibits currents that quickly desensitize while α3L shows almost no desensitization. When this same 15-residue cassette was inserted into the α1 subunit, it similarly caused a substantial decrease in desensitization (Langlhofer and Villmann, 2016; Lynch, 2004).

The β subunit has the most widespread distribution of any GlyR subunit, with transcripts found throughout the brain and spinal cord, both prenatally and in adults (Lynch, 2004; Webb and Lynch, 2007). As with α1, expression levels of the β subunit

are lower early in development, and increase significantly after birth. An 18-residue gephyrin binding domain in the TM3-4 loop has been shown to concentrate heteromeric GlyRs at postsynaptic locations (Lynch, 2004; Meyer et al., 1995).

### 1.2.3 - Activation, Gating and Desensitization

Glycine receptors are activated by multiple amino acids in the following order, by potency: glycine, β-alanine, taurine, alanine, serine, proline and GABA (Betz, 1987). Taurine (2-aminoethane sulfonic acid) is one of the most abundant amino acids in muscle tissue and organs. It is the most abundant amino acid in the neonatal brain, present at concentrations similar to glutamate in the cerebral cortex of developing rats, and is necessary for normal cortical development. By comparison, the concentration of glycine in this area is ten times lower (Flint et al., 1998). Taurine concentration in the brain decreases during later development. In adult rats, taurine levels are ~25% of those found in newborn animals (Agrawal et al., 1971). Despite this, it remains the second most abundant neurotransmitter in the adult brain where extracellular concentrations are in the millimolar range (Mori et al., 2002).

Taurine is synthesized from cysteine and methionine within the body with cysteinesulfinic acid decarboxylase (CSD) performing the rate limiting step. Not surprisingly, CSD is found in numerous tissues such as the liver, kidney, reproductive organs and brain, highlighting the importance of taurine in these areas. CSD levels are relatively low humans and other primates but taurine is readily available in diets containing meat and seafood (Ripps and Shen, 2012). A typical American diet provides

~123-178 mg of taurine per day compared to ~17 mg in a vegetarian diet. As a result, circulating taurine levels in vegetarians are lower than in their meat-eating counterparts (Caine and Ceracioti, 2016).

Although taurine is not incorporated into proteins, it performs numerous other functions. Taurine has anti-inflammatory and cytoprotective properties and acts to regulate cell volume and intracellular calcium levels. Taurine deficiency has been liked to cardiomyopathy, renal dysfunction, pancreatic β cell malfunction, retinal damage and developmental abnormalities in the brain (Caine and Geracioti, 2016; Ripps and Shen, 2012).

Glycine is generally considered to be the primary endogenous agonist of the glycine receptor; however, there is increasing evidence that the taurine may also be an important agonist at GlyRs in the CNS (Albrecht and Schousboe 2005; Ericson et al., 2006; Flint et al., 1998; Wang et al., 2005). This is supported by the research of Mori et al. (2002) who showed that inhibiting taurine transporters with guanidinoethanesulfonic acid (GES) induced a strychnine-sensitive chloride current in hippocampal organotypic slice cultures, suggesting that taurine may play a role in maintaining tonic inhibition in this region. Wang et al. (2005) later identified taurine-sensitive GlyRs on a subset of dopamine neurons in the VTA of rats which are also subject to tonic inhibition.

Taurine is considered a partial agonist at the GlyR with approximately 5% of the efficacy of glycine (Lape et al., 2008). At saturating concentrations, the probability of finding a fully-liganded α1β GlyR receptor in the open state is ~54% for taurine and 96%

for glycine, despite the fact that opening and shutting rates are similar for the two agonists (Lape et al., 2008). In early models, partial agonism was believed to be a result of the reduced ability of the bound channel to transition to the open conformation once bound by agonist. Lape et al. (2008) proposed a new model, based on single channel recordings of taurine- and glycine-activated GlyR in HEK293 cells. They postulated the existence of an additional transition state, called the 'flipped' state, that occurs after ligand binding and precedes channel opening. In this model, the lower efficacy of a partial agonist is due to having a lower equilibrium constant for entering this pre-open 'flipped' state (Figure 1.2). They demonstrated this experimentally, showing that glycine has a much higher equilibrium constant for flipping than taurine (Figure 1.2 from Lape et al, 2008). Once in the 'flipped' conformation, however, both glycine- and taurine-bound channels open at similar rates.

Strychnine is a convulsive alkaloid from the *Strychnos nux vomica* plant and competitive inhibitor of GlyRs with high (nM) affinity for all GlyR isoforms (Betz, 1987; Langosh et al., 1990; Webb and Lynch, 2007). This property was exploited early on to purify GlyRs from tissue preparations and to differentiate glycinergic from GABAergic activity (Betz et al., 1993; Lynch, 2004). Strychnine binds between residues 170 and 220 of the $\alpha$1 subunit and shares an overlapping but non-identical binding site with GlyR agonists (Kuhse et al., 1995).

Picrotoxin (PTX), present in plants in the moonseed family, consists of an equal mix of picrotin and picrotoxinin and is a potent non-specific inhibitor of anion-

conducting CLRs. At $GABA_A$Rs, PTX acts as a pore blocker but functions as an allosteric inhibitor at α subunit-containing GlyRs. PTX sensitivity is determined by the 2' and 6' residues in the pore-forming M2 domain. Due to sequence differences between α and β subunits in this area, the PTX sensitivity of heteromeric GlyRs is 50-100x lower than homomeric receptors, making it a useful tool for detecting the presence of the β subunit (Betz, 1993; Webb and Lynch, 2007).

Voltage-clamp fluorometry experiments performed on α1 GlyR expressed in oocytes were used to elucidate the structural changes that occur during agonist and antagonist binding and subsequently, signal transduction. Pless et al. (2009a,b) measured agonist-induced changes in response to activation by glycine, taurine and β-alanine, agonists that differ in efficacy, during the transition between the closed and 'flipped' states. Conformational changes caused by glycine and strychnine were also compared. They observed movements of Loops C and F and the pre-M1 domain that were indistinguishable between agonists. Loop 2 also displayed agonist-induced changes in conformation in the environment of A52, however, the magnitude of these changes were unequal and directly proportional to the efficacy each agonist. This further supports the findings of Crawford et al. (2008) who employed cysteine-scanning mutagenesis to determine that that structural differences in Loop 2 had significant, and sometimes opposite effects on glycine sensitivity. Taken together, these studies provide evidence of agonist-specific differences in a signal transduction mechanism that involves the Loop 2 domain. Differences were also observed between glycine and strychnine at loops 2, D, E

and the pre-M1 domain indicating that structural rearrangements in these regions may be involved in receptor activation.

Further advances in our understanding of GlyR activation came from the Cryo-EM structures of the zebrafish α1 homomeric glycine receptor bound to glycine and strychnine, published by Du and colleagues (2015). Following binding of agonists to the ECD, the upper portion of the ECD twists anticlockwise relative to the pore axis with the lower region tilting inwards toward the center of the pore. This motion causes significant displacement of Loop C inward, such that it closes over the ligand binding pocket and displaces the β8-β9 loop which is, in turn, connected to the pre-M1 loop causing it to also shift. This leads to rotation of the M1 helix which is connected to the intracellular portion of M2. As a result, the lower portion of M2 is pushed inward towards the pore axis. Movement of the β8-β9 loop also shifts the cys-loop. This allows the M2-M3 linker to interact with the β1-β2 loop in the ECD which forces the upper region of M2 outwards such that the extracellular half of the TMD is wider than the intracellular portion. This interaction, involving proline 291 and threonine 70, helps to stabilize the receptor in the open state. Overall, this results in a 49˚ tilt in the outer portion of the M2 helix relative to the closed conformation. Expansion of the pore causes the rotation of a ring of leucine residues at the 9' position away from the pore axis, widening the channel to up to 4 or 5 Å. In the open state, the greatest constriction point occurs at the -2' position which has a radius of 4.4 Å (Du et al., 2015).

Desensitization occurs when a receptor with a previously open channel enters a non-conducting conformation, despite the continued presence of bound agonist. The rate at which the receptor transitions to, and recovers from, a desensitized state can have profound effects on overall function, including the frequency and amplitude of glycinergic currents. The general mechanism believed to be responsible for desensitization in Cys-loop receptors involves interactions between amino acids in TM2 and TM3 nearer the intracellular region of the channel, between the 2' and 9' positions, that constitute a specific 'desensitization gate' that is largely influenced by the intracellular domains of the receptor (Gielen et al., 2015, Lynch, 2004, Nys et al., 2013). For example, the long isoform of the α3 subunit, α3L, contains a 15-residue splice cassette in the intracellular domain which renders these receptors largely insensitive to desensitization. Point mutations in the TM1-TM2 of α1 GlyRs, found in some cases of human hyperekplexia, such as I244N and P250T, have the opposite effect (Lynch, 2004).

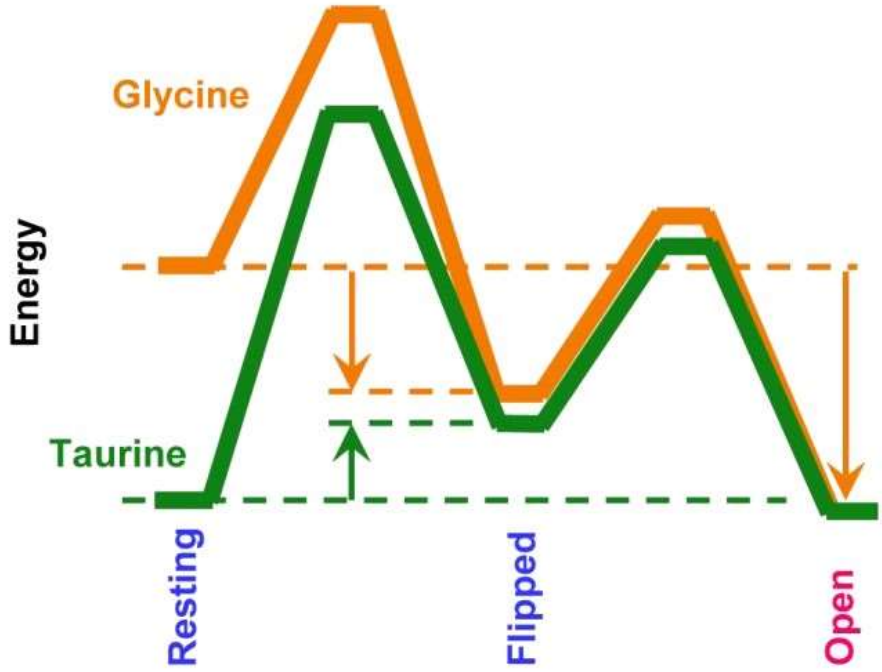


**Figure 1.2: Model of the 'flipped' mechanism for partial agonism**

Energy diagrams of the transitions between the closed, flipped, and open states. Taurine has a much higher energy barrier for reaching the flipped conformation than glycine but the subsequent transitions from flipped to open are similar. Adapted from *Lape, R., Colquhoun, D., Sivilotti, L.G., 2008. On the nature of partial agonism in the nicotinic receptor superfamily. Nature. 454, 722-727.*

### 1.2.4 - Intracellular Domain

Regions of the large intracellular loop (IL) connecting TM3 to TM4 helps determine the properties of specific GlyR subunits. The scaffolding protein gephyrin interacts with an 18 residue portion of the β subunit anchoring the receptor to cytoskeletal filaments (Meyer et al., 1995). Gephyrin is also a key mediator of receptor trafficking through interactions with the microtubule-associated motor proteins KIF5 and dlc1/2. Similarly, a conserved poly-proline helix type II recognition motif, $^{384}$KxxPxxPxxP$^{394}$ found in the β subunit, interacts with Syndapin which is also involved in receptor trafficking (Langlhofer and Villmann, 2016). Thus, the IL plays a critical role in GlyR surface expression and the formation of inhibitory synapses. The 15-amino acid insertion in the IL of α3L helps targets these subunits to presynaptic sites through interactions w/ the vesicular trafficking protein Sec8. Mutations in the TM3-TM4 loop that are linked to defects in receptor trafficking and subsequent accumulation in the ER and Golgi have been found in cases of recessive hyperekplexia (Langlhofer and Villmann, 2016).

The GlyR-IL serves as a site for posttranslational modification including ubiquitination of lysine residues and phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and tyrosine kinases, whose effects differ depending on the GlyR subtype and location (Burgos et al., 2015). Increased PKA activity in cultivated brain stem neurons enhances glycine-evoked currents (Kuhse et al., 1993). PKC-dependent phosphorylation of S391 in the α1 subunit increases the rate of GlyR internalization by endocytosis. Phosphorylation of S403 in the β subunit impairs the ability of the receptor

to bind gephyrin, causing a decrease the synaptic GlyR density by diffusion away from the synapse. The α3 subunit also possess a unique PKA consensus sequence involving S346. Phosphorylation of this site causes receptor internalization which contributes to $PGE_2$-mediated inhibition of glycinergic neurotransmission known to contribute to inflammatory pain sensitization (Langlhofer and Villmann, 2016).

Stretches of highly conserved basic amino acids located near the pore affect ion selectivity and conductance and may be involved in the rearrangement of M3 and M4 during channel gating. There is also evidence that $^{316}$RFRRK$^{320}$ and $^{385}$KK$^{386}$ in the GlyR-IL of the α1 subunit interacts with Gβγ which may be critical for the enhancing effects of ethanol at these receptors. K385 in this region has also been linked to allosteric modulation of GlyRs by endocannabinoids (Langlhofer and Villmann, 2016).

### 1.3 - GlyR Modulation

GlyR function is affected by a variety of endogenous compounds such as zinc, glutamate, endocannabinoids and neuroactive steroids (Yevenes, et.al., 2011) as well as numerous exogenous modulators including alcohols, anesthetics, inhalants and cannabinoids that interact with regions of the receptor outside of the orthosteric agonist binding site (Figure 1.3) (Beckstead et al., 2000; Harvey et al., 1999; Kirson et al., 2013, 2012; Mascia et al. 1996; Mihic et al., 1997, Xiong et al., 2011). Allosteric modulators shift glycine concentration-response curves either to the left or to the right but have negligible effects at maximally-effective glycine concentrations. Work in our lab, however, determined that ethanol, volatile anesthetics, inhaled drugs of abuse and zinc

are able to enhance GlyR currents elicited by maximally-effective concentrations of the partial agonist taurine (Kirson et al. 2013a, 2012b). This suggests that the mechanism of allosteric modulation of GlyRs depends on whether the receptor is activated by glycine or taurine.

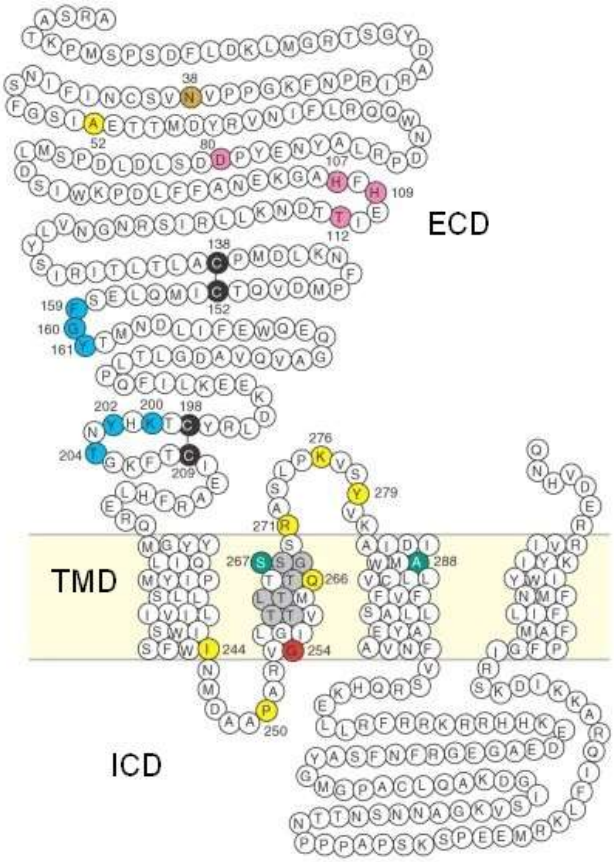


**Figure 1.3: Residues involved in glycine receptor activation and modulation**

Illustration of a single α1 GlyR subunit embedded in the plasma membrane showing the location of important residues involved in activation and modulation of the receptor in the extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD). Black and brown circles represent cysteine residues forming disulfide bonds and the N-glycosylation site, respectively. Locations of naturally occurring murine *spasmodic* and human hyperekeplexia mutations are highlighted in yellow. Blue residues are involved in binding agonists and antagonists. Residues involved in the actions of alcohols and anesthetics are shown in green while those involved in zinc modulation are pink. G254 (red) determines pore conductance and sensitivity to pore blockage by cyanotriphenylborate (CTB). Grey residues in M2 are believed to line the pore. Adapted from *Laube, B., Maksay, Gl., Schemm, R., Betz, H., 2002. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses. TRENDS Pharmacol. Sci. 23, 519-527.*

### 1.3.1 - Inhalants and Anesthetics

Anesthetics and inhaled drugs of abuse act as positive allosteric modulators of GlyR and $GABA_AR$ function. Isoflurane increased the frequency of mIPSCs in rat trigeminal nucleus and spinal motoneurons and reduced the firing of spontaneous action potentials in rat spinal slice cultures (Burgos et al., 2016, 2015). The binding site for volatile anesthetics such as isoflurane, enflurane, halothane and sevoflurane is located in an intrasubunit cavity in the transmembrane domain of GlyRs and $GABA_ARs$. Mihic et al. (1997) created chimeric receptors using the GlyR α1 subunit, which is positively modulated by anesthetics and alcohols, and the homologous ρ1 $GABA_CR$, which is inhibited by these compounds, to identify a 45 residue stretch of amino acids in the transmembrane domain of these receptors that confers sensitivity to ethanol, enflurane and isoflurane. They then utilized site-directed mutagenesis to pinpoint S267 in TM2 and A288 in TM3 of the α1 GlyR (and corresponding residues in $GABA_AR$) as critical for these actions. Beckstead et al. (2002) expanded on this work to show that the site of action of inhalants such as TCE, TCY and Toluene overlap with the same binding pocket as volatile anesthetics including the serine residue at position 267.

### 1.3.2 - Cannabinoids

More recently, GlyRs but not $GABA_ARs$ have been identified as targets for cannabinoids. At physiologically relevant concentrations, the endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG) act directly on GlyRs in the hippocampus to modulate neuronal activity (Lozovaya et al., 2005). Hejazi et al. (2006)

then demonstrated that AEA and Δ9-tetrahydrocannabinol (Δ9-THC) are capable of potentiating glycine-evoked GlyR currents in neurons isolated from the rat ventral tegmental area (VTA) as well as α1 homomeric and α1β heteromeric GlyRs, activated by low (< 30μM) concentrations of glycine, expressed in *Xenopus laevis* oocytes. In both studies, these effects were shown to be completely independent of cannabinoid GPCR activity. Xiong et al. (2011) used mutagenesis to identify a serine residue at position 296 in α1 and an equivalent serine in α3 that is necessary for higher Δ9-THC sensitivity. They conferred similar sensitivity to the α2 subunit by mutating the corresponding alanine residue at position 303 to serine. Performing the reverse mutation in α1 and α3 significantly impaired modulation by Δ9-THC.

### 1.3.3 - Neuroactive steroids

A number of neuroactive steroids are known to vary in their effects on different GlyR subunits. Pregnenolone (PREG) potentiates α1 GlyR but has no effect on α2 or heteromeric receptors. The synthetic neurosteroids, minaxolone, alphaxalone and Org20599 enhance recombinant α1 GlyR activity (Yevenes et al., 2011). In contrast, pregnenolone sulfate (PREGS) and dehydroepiandrosterone (DHEAS) are inhibitory, with greater effects at α1 homomeric receptors than heteromeric or α2 homomeric GlyR, while progesterone shows α2-specific inhibition and acts at site distinct from that of PREGS (Betz and Laube, 2006; Webb and Lynch, 2007).

### 1.3.4 - Ethanol

Excessive alcohol use is a global health problem and is estimated to be a contributing factor in ~3.8% of deaths, world-wide. The costs associated with this account for more than 1% of the gross national product (GNP) in middle to high-income countries (Rehm et al., 2009). In the United States, excessive alcohol consumption is the 4$^{th}$ leading preventable causes of death, accounting for 1 in 10 deaths in working-age adults. Between 2006 and 2010 an average of 87,798 people died from excessive alcohol use per year and 2.5 million years of potential life were lost (Stahre et al., 2014). The economic burden of excessive drinking in the U.S. was $248 billion in 2010 alone, with approximately 77% of these costs attributed to binge drinking (Sacks et al., 2015).

Some of the pharmacological effects of ethanol are believed to be due to its actions as a positive allosteric modulator of glycine and $GABA_A$ receptors. Ethanol concentrations of 50mM or more consistently enhance GlyR currents elicited by taurine and sub-saturating concentrations of glycine (Webb and Lynch, 2007). Two putative alcohol binding sites per subunit have been identified on the GlyR. S267 and A288 in the transmembrane domain of α1 GlyR form part of a binding pocket that is critical for the potentiating actions of alcohols and anesthetics (McCracken et al., 2016; Mihic et al., 1997). Mutations in neighboring residues also have substantial effects on ethanol modulation of GlyRs. Exchanging Q266 in TM2 with isoleucine abolished EtOH potentiation and significantly reduced agonist sensitivity. M287L in TM3 also caused a significant reduction in EtOH potentiation (Borghese et al., 2012). The consecutive

amino acids, I409, Y410 and K411 which are believed to form a hydrophilic cavity in TM4 are also critical determinants of EtOH modulation (Burgos et al., 2015).

It has been proposed that the A52-containing Loop 2 region of the α1 GlyR forms a second binding site for ethanol (Naito et al., 2014; Perkins et al., 2012, 2008). This confirms an earlier report by Mascia et al. (1996) showing that the potentiating effects of ethanol on glycine-evoked currents were attenuated in the murine *spasmodic* mutant, $\alpha1^{A52S}$. These studies, however, have focused on glycine-activated GlyR. To our knowledge, no one has characterized the effects of taurine on GlyR Loop 2 mutants.

Ethanol differentially modulates different GlyR subtypes, having substantially greater effects on α1 GlyRs compared to α2 and α3. In the α2 subunit this difference has been mapped to a threonine residue in the Loop 2 domain. In contrast, the α1 subunit contains an alanine at position 52. Ethanol resistance in the α3 subunit is conferred by the 15-amino acid cassette in the IL discussed in greater detail above (Burgos et al., 2015).

Two conserved basic amino acid motifs in the GlyR intracellular loop, $^{316}$RFRRK$^{320}$ and $^{385}$KK$^{386}$ are critical for a number of processes including membrane insertion, ion selectivity, nuclear import and interactions with G-protein Gβγ subunits. The latter function has been shown to enhance glycine-evoked currents at heterologously expressed GlyRs (Langlhofer and Villmann, 2016). A number of studies have been published implicating the intracellular loop and its interactions with G-proteins in the

potentiating effects of ethanol (Castro et al., 2012; San Martin et al., 2012; Yevenes et al., 2010).

Yevenes et al. (2008) used alanine scanning experiments to identify amino acid sequences in the GlyR-IL that are critical for ethanol potentiation of α1 GlyRs. They found that single and double alanine substitutions within two stretches of basic residues, $^{316}$RFRRK$^{320}$ and $^{385}$KK$^{386}$, significantly attenuated ethanol potentiation of GlyR currents. San Martin (2012) and colleagues later blocked this interaction with a series of peptides based on residues 309-325 from the N-terminal portion of the IL in cultured spinal neurons, causing a concentration dependent inhibition of EtOH potentiation. K385A/K386A knock-in mice exhibited a 30% shorter loss of righting reflex (LORR), demonstrating that amino acid identity at these positions are also important for the effects of ethanol on motor control (Burgos et al., 2015).

Single channel analysis of glycine-activated WT human α1 GlyRs expressed in oocytes revealed that EtOH increases durations of bursts and the number of openings per burst but had no effect on the percentage of time the channel spends in the open state within a burst or the likelihood of entering a given burst state (Welsh et al., 2009). The model constructed from these data suggest that EtOH increases glycine affinity for the receptor by antagonizing glycine unbinding. Subsequent experiments of taurine-gated receptors led to the initial hypothesis that EtOH acted via a similar mechanism (Welsh et al., 2010). Work by Kirson and colleagues (2012), however, showed that EtOH

potentiates GlyR currents elicited by maximally effective concentrations of taurine and must, therefore, also act to enhance taurine efficacy.

The mesolimibic dopamine reward pathway, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (nAc), limbic system, and orbitofrontal cortex, mediates the reinforcing effects of drugs of abuse (Enoch, M., 2008). It is through this pathway that glycine receptors are believed to mediate drug-related behavior (Blednov et al., 2015; Jonsson et al., 2014; Li et al., 2012; Molander et. al, 2005; Ye et. al, 2001). There is increasing evidence for the role of GlyRs in the regulation of DA release in the nAc in response to alcohol. Ye et al. (2001) identified alcohol-sensitive glycine receptors in dissociated VTA neurons from rats. It was later shown that microdialysis of glycine into the nucleus accumbens of ethanol-preferring Wistar rats increased the levels of dopamine in this region (Ericson et al., 2006; Molander et al., 2005). This same glycine treatment also caused a decrease in ethanol preference and consumption (Molander et al., 2005). Male Wistar rats treated with ORG25935, an inhibitor of glycine transporter-1, also decreased their ethanol intake (Molander et al., 2007). Similarly, microinjection of glycine into the ventral tegmental area (VTA) decreased ethanol consumption in Long-Evans rats (Li et al., 2012).

Acamprosate, a synthetic drug derived from homotaurine, is one of the few pharmacological interventions currently available for the treatment of alcohol addiction and is used to prevent relapse in abstinent alcoholics. Chau et al. (2010) found that systemic treatment with acamprosate caused a decrease in voluntary alcohol intake in

medium- and high-alcohol-preferring rats, a behavior that was reversed by microinjection of strychnine into the nAc. This suggests that the therapeutic effects of acamprosate may be mediated, at least in part, through GlyRs in the nAc and highlights their potential as a target for the treatment of addiction.

### 1.3.5 - Zinc

The divalent cation, zinc is ubiquitous in the CNS where it is crucial for proper brain development and function in animals and humans. The majority of zinc in the brain is bound to proteins, leaving 10% as "free" chelatable zinc. Some of this is packaged into presynaptic vesicles by members of the SLC30 superfamily of zinc transporters and can be released into the synapse in a calcium-dependent manner, along with neurotransmitters such as glycine and glutamate (Hirzel et al., 2006, Webb and Lynch, 2007; Trombley et al., 2011).

Zinc is a biphasic modulator of GlyR function. Concentrations <10 μM potentiate GlyR currents while levels that surpass 10 μM are inhibitory. Tonic zinc concentrations have been estimated to be anywhere from 5-50 nM in human cerebral spinal fluid (CSF) (Fredrickson et al., 2006a) to as high as 200 nM (Hirzel et al., 2006). Synaptic release, however, temporarily results in much higher concentrations, potentially in the inhibitory range, with earlier estimates ranging from <10 μM to 100 μM or more (Fredrickson et al., 2006b; Qian and Noebels, 2005; Vogt et al., 2000). More recently, Zhang et al. (2016a) used artificial synapses as a model to measure zinc concentrations following synaptic

release. Their work suggests that synaptic zinc may reach levels of 1 μM or more and are likely higher at glycinergic than glutamatergic synapses.

The biphasic action of zinc on GlyRs is attributable to two types of binding sites on these receptors. At low concentrations, zinc occupies high affinity binding sites on the outer surface of the N-terminal domain which causes potentiation of GlyR currents by enhancing agonist affinity for the receptor. At higher concentrations, the high affinity binding sites are saturated and zinc also associates with lower affinity inhibitory binding sites on the inner side of the ECD, facing towards the vestibule (Burgos et al., 2016). Although both the α1 and α2 subunits have nanomolar affinity for zinc, α2 GlyR are 15x less sensitive to zinc potentiation. It was determined that a single amino acid difference, D194 in α1 and E201 in α2, between the two subunits is responsible for this disparity. Mutating D194 to alanine abolished zinc potentiation of both glycine- and taurine-evoked currents, confirming that this residue is necessary for the enhancing effects of zinc (Miller et al., 2005).

Additional residues have also been implicated in zinc potentiation. The L274A mutation in the TM2-TM3 loop caused a reduction in glycine and taurine affinity while eliminating the potentiation of glycine- but not taurine-activated currents, suggesting that zinc has agonist-specific effects on GlyR function. The intracellular M246A and D80A mutations were also reported to disrupt enhancement of glycine-evoked currents (Lynch et al., 1998), however mutations at D80 do not appear to affect zinc enhancement of taurine currents (Miller et al., 2005). A more recent study, however, found no disruption

of zinc enhancement of glycine-activated currents in α1 D80A GlyRs expressed in oocytes and only partial attenuation of zinc effects at D80G (Cornelison et al., 2017). The α1 W170S is a gain-of-function, missense mutation in Loop F that causes an autosomal recessive form of human hyperekplexia and has been found to completely abolish zinc potentiation of glycine, taurine and β-alanine currents, possibly by disrupting the nearby zinc binding site (Zhang et al., 2016b; Zhou et al., 2013).

The zinc-coordinating actions of H107 and H109 at the inhibitory binding site in α1 homomeric GlyRs was demonstrated by Harvey et al. (1999) who showed that deprotonating these residues at pH 5.4 was sufficient to disrupt zinc inhibition. Their importance was further validated through mutagenesis. A single histidine to alanine mutation at either H107 or H109 caused a significant decrease in zinc inhibition while the H107A/H109A double mutant was completely insensitive to the inhibitory effects of zinc (Harvey et al., 1999). This also explains the lower sensitivity of α2 and α3 GlyRs to zinc inhibition as both subunits contain an asparagine residue at the site corresponding to H107 in the α1 subunit (Webb and Lynch, 2007). A model based on the structure of AChBP showed that H107 and H109 are likely to be located near subunit interfaces (Laube, 2002). It was later shown that these histidines form part of an intersubunit zinc binding site between H107 located on the (+) face and H109 and T133 on the (-) face which are believed to help stabilize the receptor in the closed state (Nevin et al., 2003).

The actions of zinc on GlyRs are also important for glycinergic neurotransmission *in vivo*. Homozygous D80A knock-in mice exhibited severe neuromotor defects, similar

to those found in the *spasmodic, spastic* and *oscillator* murine models of hyperekplexia, including an enhanced startle response, inducible tremors and impaired motor performance (Hirzel et al., 2006). Zinc has also been shown to act synergistically with ethanol, but not pentanol or isoflurane, to further potentiate GlyR currents (McCracken et al., 2010). Zinc sensitivity also seems to affect ethanol-related behavior. McCracken et al. (2013) found that C57BL/6 mice expressing the D80A mutation in the α1 subunit of the GlyR showed a reduction in ethanol consumption and preference.

## 1.4 - Channelopathies

Glycine receptor dysfunction is implicated in multiple diseases. Pre-synaptic $\alpha3^{P185L}$ GlyRs at glutamatergic terminals contribute to hyperexcitability and cognitive impairment in patients suffering from temporal lobe epilepsy (Langlhofer and Villman, 2016). There is also evidence that defects in the GlyR α2 subunit contribute to the pathology of Autism Spectrum Disorders (ASDs). A rare human X-linked microdeletion of exons 8 and 9 encoding the TM3-4 loop in the *Glra2* has been associated with autism. This variant was shown to cause a decrease in surface expression *in vitro* and severe defects in axon-branching in zebrafish. *Glra2* KO mice exhibited deficits in object recognition memory as well as impairment of long-term potentiation (LTP) in the pre-frontal cortex (Langlhofer and Villman, 2016).

### 1.4.1 - Hyperekplexia

Disruption in glycinergic function is the major cause of the heritable neurological disorder, hyperekplexia. Symptoms include an exaggerated startle response to

unexpected stimuli causing severe muscle rigidity, loss of postural control, tremors and apnoea. This is most often treated with benzodiazepines, such as clonazepam, which increase GABergic activity (Lynch, 2004; Schaefer t al., 2013).

The most common forms result from mutations in genes encoding GlyR subunits, however, some cases arise from mutations in *SLC6A5*, encoding the presynaptic glycine transporter 2 (GlyT2), and even more rarely, proteins responsible for anchoring synaptic GlyR such as gephyrin and collybistin (CB) (Langlhofer and Villmann, 2016). Dominant hyperekplexia mutations tend to be located in the ion channel domain, causing defects in GlyR function. Recessive mutations are more widely distributed and can affect surface expression, trafficking and receptor stability (Chung et al., 2010; Langlhofer and Villmann, 2016).

Murine mutants exhibiting hyperekplexia-like phenotypes are common models of the human disorder. The *oscillator* mutation is caused by a 7 base-pair deletion in exon 8 of the α1 subunit that results in a premature stop codon. Mice that are homozygous for this mutation lack α1-containing GlyR in the brainstem and spinal cord (Graham et al., 2006). Phenotypically, they start exhibiting symptoms at postnatal day 14 and die by postnatal day 21 (Langlhofer and Villmann, 2016). The insertion of a LINE 1 transposable element in the gene encoding the GlyR β subunit causes exon skipping which results in decreased transcription efficiency in *spastic* mice. The α1 A52S substitution in Loop 2 of the ECD is found in the mouse *spasmodic* mutant. These receptors show decreased agonist sensitivity similar to the α2 subunit (Graham et al.,

2006). This mutation is of particular interest as it has also been shown to have a significant effect on ethanol sensitivity (Mascia et al., 1996).

### 1.4.2 - Inflammatory Pain Sensitization

α3-containing GlyRs are highly expressed in laminae I and II of the spinal cord dorsal horn where they act to regulate pain signaling (Betz and Laube, 2006). Heteromeric α3β receptors are particularly significant for inflammatory pain perception (Imlach et al., 2016). The release of prostaglandin $E_2$ ($PGE_2$) increases PKA-dependent phosphorylation of S346 in the TM3-4 loop of the α3 subunit, causing a reduction in glycinergic signaling. This results in decreased inhibition of nociceptive projection neurons, causing enhanced pain sensitization (Acuna et al., 2016; Lynch, 2009; Langlhofer and Villmann, 2016; Webb and Lynch, 2007). Based on this mechanism, α3 GlyRs present a promising target for pain management.

Pharmacological agents targeting α3 receptors have already been shown to alleviate hyperalgesia in rodent models. The analgesic effects of Δ9-THC have been demonstrated in WT, $\alpha2^{-/-}$, $CB1^{-/-}$ and $CB2^{-/-}$ but not $\alpha3^{-/-}$ C57BL/6J mice, indicating that these effects are mediated by α3 GlyRs (Xiong et al., 2011). Administration of cannabidiol and its derivative, dihydroxyl-CBD (DH-CBD), similarly reduced pain sensitization and perception in rats and specifically reversed $PGE_2$-induced pain sensitization in mice (Xiong et al., 2011). Acuna et al., 2016 later showed that 2,6-di-ert-butylphenol (2,6-DTBP), a propofol derivative with μM affinity for phosphorylated α3β GlyRs, caused a significant reduction of induced hyperalgesia in mice.

# Chapter 2: Materials and Methods

General experimental procedures and materials are described below. Further information regarding specific experiments pertinent to each study can be found within the materials and methods section of the relevant chapter.

## 2.1 - Buffers and Reagents

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) with the exception of tricaine which was purchased from Western Chemical, Inc. (Ferndale, WA). The following buffers were made using ultra-pure $H_2O$. pH was adjusted, as necessary, with NaOH or HCl.

**Barth's saline (MBS):** 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.5

**Isolation Medium:** 108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES, pH 7.5

**Incubation Medium:** MBS with 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin, and 50 mg/l gentamicin, pH 7.5

**Collagenase Solution:** 83 mM NaCl, 2 mM $MgCl_2$, and 5 mM HEPES, 0.5 mg/ml Sigma Type 1A collagenase, pH 7.5

## 2.2 - Site-Directed Mutagenesis

Point mutations were generated via site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and commercially engineered primers (Integrated DNA Technology, San Diego, CA)

using a wildtype (WT) glycine receptor cDNA template in a modified pBK-cytomegalovirus vector containing a gene conferring resistance to the antibiotic kanamycin (Mihic et.al., 1997).

Forward and reverse primers were combined with 5-50 ng of the GlyR template, reaction buffer, deoxynucleotide triphosphate mix and *PfuTurbo* DNA polymerase (2.5 U/μL) and run through the thermocycling protocol specified by the manufacturer. Each reaction was then combined with 1 μL of the Dpn I restriction enzyme (10 U/μL) and incubated for 1 hour at 37C to digest the template DNA.

The resulting plasmids were transformed into XL1-Blue Supercompetent *E. coli* cells (Agilent Technologies, Santa Clara, CA) and then grown on agar plates that contained 50 μg/mL kanamycin to allow for selection of cells containing plasmid DNA. Individual colonies were then selected and grown overnight in LB broth in a shaker incubator set at 37C and 225-250 rpm.

Plasmids were isolated using QIAprep® Spin Miniprep Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). cDNA concentration and quality was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples with an $A_{260/280}$ of 1.8-2.0 and an $A_{260/230}$ of > 1.8 were deemed suitable for injection into oocytes. Mutations were verified through Sanger sequencing at the University of Texas at Austin DNA Sequencing Facility.

### 2.3 - Oocyte Isolation and Injection

Female *Xenopus laevis* frogs were procured from Nasco (Fort Atkinson, WI) and housed at 19C on a 12-hour light/dark cycle. Frogs were anesthetized for 10 minutes in a tricaine solution before performing partial ovariectomies under an approved IACUC animal protocol. Portions of the ovary were removed through a 1 cm incision in the lower abdomen and stored in incubation medium until isolation. The incision was closed with sutures and the frogs were allowed to recover for at least 3 hours in a separate container before being returned to their normal housing. Each animal was allowed to recover for at least 4 weeks between surgeries.

Stage V and VI oocytes were placed in a hypertonic isolation medium in order to make oocytes isolation easier. Oocytes were isolated manually with forceps to remove the thecal and epithelial layers and temporarily stored in incubation medium that was sterilized by passage through a 0.22-μm filter. The follicular layer was removed by placing isolated oocytes in collagenase buffer for 10 minutes. Following collagenase treatment, oocytes were placed in incubation medium where they remained throughout the injection process.

Oocytes were injected with 32.2 nL of human WT or mutant glycine receptor subunit cDNA at a concentration of 50 ng/μL via the "blind" method of Colman (1984) using a micropipette (10-15 μm tip size) attached to an electronic microdispenser. Oocytes were then placed, individually, in 96 well plates containing incubation medium and stored in the dark at room temperature prior to use.

### 2.4 - Two-electrode Voltage-Clamp Electrophysiology

Oocytes expressed GlyRs within 24-48 hours. All electrophysiology measurements were made 1-5 days following cDNA injection. Oocytes were placed in a 100 μL bath with their animal poles facing upwards. The animal pole of each cell was pierced with two high-resistance (0.5–10 MΩ) glass electrodes filled with 3 M KCl and voltage-clamped at -70 mV using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT). One electrode was used to constantly monitor the voltage across the membrane while the other was used to inject negative current into the cell to maintain the clamp.

Oocytes were perfused with MBS at a rate of 2 mL/min using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) through 18-gauge polyethylene tubing. All solutions were prepared in standard MBS or MBS containing 2.5 mM tricine. Oocytes were preincubated with modulators for 30-60s prior to co-application with agonist. Agonist applications lasted 30s for submaximal concentrations and 15-30s for maximally-effective concentrations. Washout periods ranged from 3-15 minutes, depending on the concentration of agonist used.

GlyR activation in this paradigm resulted in the outward flow of chloride ions. The current electrode was used to inject negative current into the cell, proportional to this chloride efflux, counteracting the resulting change in membrane potential. The amount of current needed to maintain the voltage clamp was continuously monitored with a Powerlab 4/30 digitizer with LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia) and used later in data analysis.

# Chapter 3: Allosteric modulation of Glycine Receptors Activated by Agonists Differing in Efficacy[1]

## 3.1 – Introduction

The glycine receptor (GlyR) is a member of the Cys-loop family of ligand-gated ion channels. It is the primary inhibitory receptor in the brainstem and spinal cord but also plays important roles in higher brain regions including the hippocampus, nucleus accumbens and prefrontal cortex (Baer et al 2009; Jonsson et al 2012, 2009; Lynch 2004). GlyRs are pentameric in structure with the 5 subunits arranged around a central anion-conducting channel. Thus far, four alpha subunits and one beta subunit have been identified of which α1-3 and β are found in humans. GlyRs express either as homomeric receptors composed solely of α subunits or as αβ heteromeric receptors with a stoichiometry of 2α:3β (Betz et al 1993; Bowery et al 2006; Lynch 2004) or 3α:2β (Grudzinska et al., 2005).

GlyR activity is affected by a large variety of allosteric modulators including zinc, alcohols, anesthetics and inhaled drugs of abuse (Beckstead et al 2000; Harvey et al 1999; Mihic et al 1997), making it a promising clinical target for the treatment of alcohol and drug addiction (Tipps et al 2010). Zinc is present endogenously at nanomolar concentrations known to enhance GlyR function. Zinc exhibits biphasic actions at

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[1]Portions of this chapter have previously been published in *Brain Research.* Farley, N-M. M., Mihic, S.J. 2015. Allosteric modulation of the glycine receptor activated by agonists differing in efficacy. Brain Res 1606, 95-101. 

GlyRs, potentiating currents at concentrations <10 mM while higher concentrations produce inhibition (Harvey et al 1999, Laube et al 2000).

Taurine is a partial agonist of the GlyR, with only 5% of the efficacy of glycine (Lape et al., 2008), and is believed to be an important GlyR agonist in a number of brain regions (Albrecht et al 2005; Mori et al 2002). Previous research has largely focused on allosteric modulation at glycine-activated receptors. Modulators shift glycine concentration-response curves either to the left or to the right but have no effects at maximally-effective glycine concentrations. However, Kirson et al. (2012, 2013) showed that ethanol, volatile anesthetics, inhaled drugs of abuse and zinc are able to enhance currents elicited by maximally-effective concentrations of taurine, but not glycine. This suggested that these modulators effect the probability of channel opening (Po), which would already be near maximum when a saturating concentration of glycine was tested. Most studies of allosteric modulation are performed using concentrations of agonists that are low on their concentration-response curves, since it is at these agonist concentrations that the greatest modulatory effects are seen. I tested whether the effect of modulator-induced increase in Po on taurine-activated GlyR would be seen when low concentrations of taurine were tested; i.e., did the magnitude of modulator enhancement differ when equi-effective concentrations of glycine and taurine were applied to GlyR?

## 3.2 – Materials and Methods

### 3.2.1 - Two-electrode voltage-clamp electrophysiology

Oocyte isolation, injection and two-electrode voltage-clamp electrophysiology experiments were completed using the methods outlined in Chapter 2 with the following additions. All solutions were prepared in one of the following: MBS, MBS + 100 nM zinc, MBS + 200 mM EtOH, or MBS + 2.5 μM zinc with 2.5 mM tricine. Drug applications lasted 30 s for submaximal glycine and taurine concentrations and 15 s when maximally-effective agonist concentrations were used. Washouts periods lasted 3-15 minutes, as appropriate, for each agonist application.

### 3.2.2 - Data Analysis

Peak currents were measured and used in data analysis. Currents observed in the presence of agonist were compared with currents elicited by co-application of agonist and modulator. Experimental values are listed as the mean ± S.E.M. Statistical significance was determined using *t*-tests, paired *t*-tests and Two-way ANOVAS and performed using SigmaPlot version 11.0 (Systat Software, San Jose, CA).

## 3.3 – Results

### 3.3.1 – Zinc enhancement of equivalent glycine and taurine currents at α1 wildtype glycine receptors

Zinc was tested for its enhancing effects of α1 homomeric GlyR currents elicited by submaximal concentrations of glycine or taurine, two agonists which differ markedly in their efficacies. Concentrations of each agonist were first identified that produced equal absolute currents, corresponding to 5 - 10% of the maximally-effective glycine

response ($EC_{5\text{-}10}$ glycine). In order to do so the $EC_{5\text{-}10}$ concentration of glycine was first identified in each oocyte and then the concentration of taurine producing a similar current was determined. Where that concentration of taurine fell on the taurine concentration- response curve was next determined, relative to a maximally-effective concentration of taurine (100 mM). A concentration of 84 ± 4 μM glycine had an EC value of 6.28 ± 0.70 relative to 10 mM glycine, while a concentration of 1.2 ± 0.2 mM taurine, producing currents of the same magnitude in each oocyte as glycine, had an EC value of 22.69 ± 3.98 relative to 100 mM taurine. The concentrations of taurine used thus fell significantly higher on their concentration-response curves than the concentrations of glycine did on theirs [$t(4) = 4.06$, $p < 0.005$] (Fig. 3.1).

I next compared the enhancing effects of 100 nM zinc on currents produced by a low concentration of glycine with zinc effects on the same absolute currents produced by the partial agonist taurine. In this experiment, zinc was co-applied with concentrations of glycine or taurine producing 5-10% of the maximally effective glycine response ($EC_{5\text{-}10}$ gly). Zinc-potentiated currents were compared to those produced by agonist alone as shown in Figure 3.2A. Co-application with zinc resulted in a significant enhancement of both glycine- and taurine-mediated currents [$F(1,23) = 24.12$, $p < 0.001$]. There was, however, no difference in the degree of zinc enhancement seen between glycine and taurine [$F(1,23) = 1.19$, $p > 0.28$].

When data from individual oocytes were plotted (Fig. 3.2B), there was a large degree of variation in zinc enhancement seen. Previous studies revealed that the buffers

used in our studies contain nanomolar levels of contaminating zinc, sufficient to affect GlyR function (Cornelison et al., 2014). I hypothesized that the degree of variability within my data set might be attributable to variations in this amount of contaminating zinc between the different preparations of buffers used in this experiment. In order to minimize the effects that variable background levels of zinc might have on my results, I repeated my previous experiment using a much higher concentration of zinc (2.5 μM) applied with a fixed concentration (2.5 mM) of the zinc chelator, tricine. This resulted in much lower inter-experiment variability in zinc enhancement but, again, no differences in zinc effects on glycine- vs. taurine-activated GlyR (Figs. 3.3A,B) [$F(1,15) = 0.62$, $p > 0.44$].

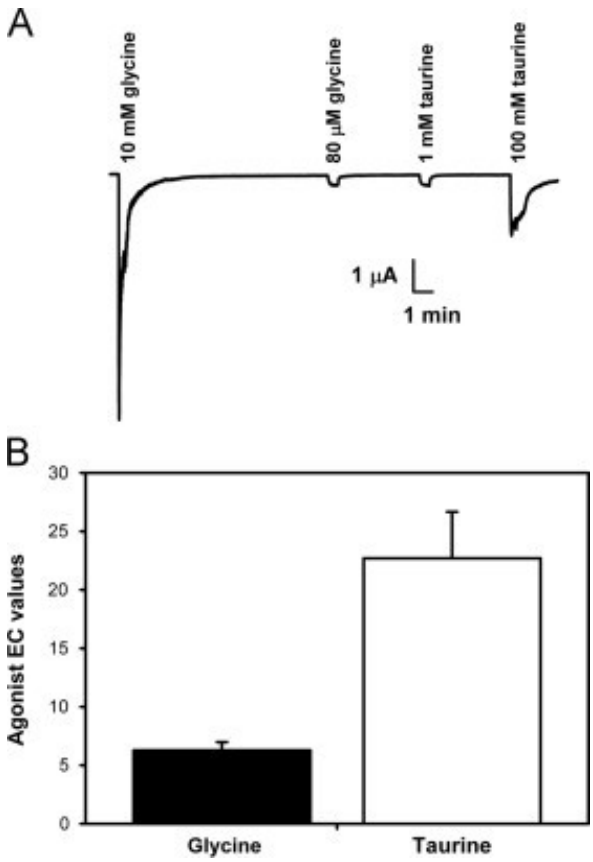


**Figure 3.1: Matching glycine and taurine currents differ in the respective EC values**

Low concentrations of glycine and taurine that elicit the same absolute currents correspond to very different effective concentrations on their respective concentration response curves. The y-axis represents the percent maximal effect for each agonist respectively, relative to their maximal currents. Data are shown as mean ± S.E.M. of 5 oocytes.

### 3.3.2 – Concentration-dependent modulation of GlyR currents by zinc

The goal of my next experiment was to determine how the percent enhancement of taurine-activated GlyR currents by zinc varied with taurine and glycine concentration. The average zinc percent potentiation as well as taurine EC value were plotted against taurine concentration (Fig. 3.4A). A concentration of 2.5 μM zinc (+ 2.5 mM tricine) enhanced taurine responses in a manner that was dependent on the concentration of taurine used [$F(5,38) = 2.55$, $p < 0.05$], with less enhancement seen at higher taurine concentrations. However, it should be noted that, even at saturating taurine concentrations, zinc still had a potentiating effect. When glycine was the agonist (Fig. 3.4B) zinc again enhanced GlyR currents in a concentration-dependent manner [$F(4,19) = 16.29$, $p < 0.001$], with less enhancement seen at higher glycine concentrations. Zinc produced no enhancement at $EC_{50}$ glycine concentrations and above, in contrast to the clear enhancement still seen at those taurine concentrations.

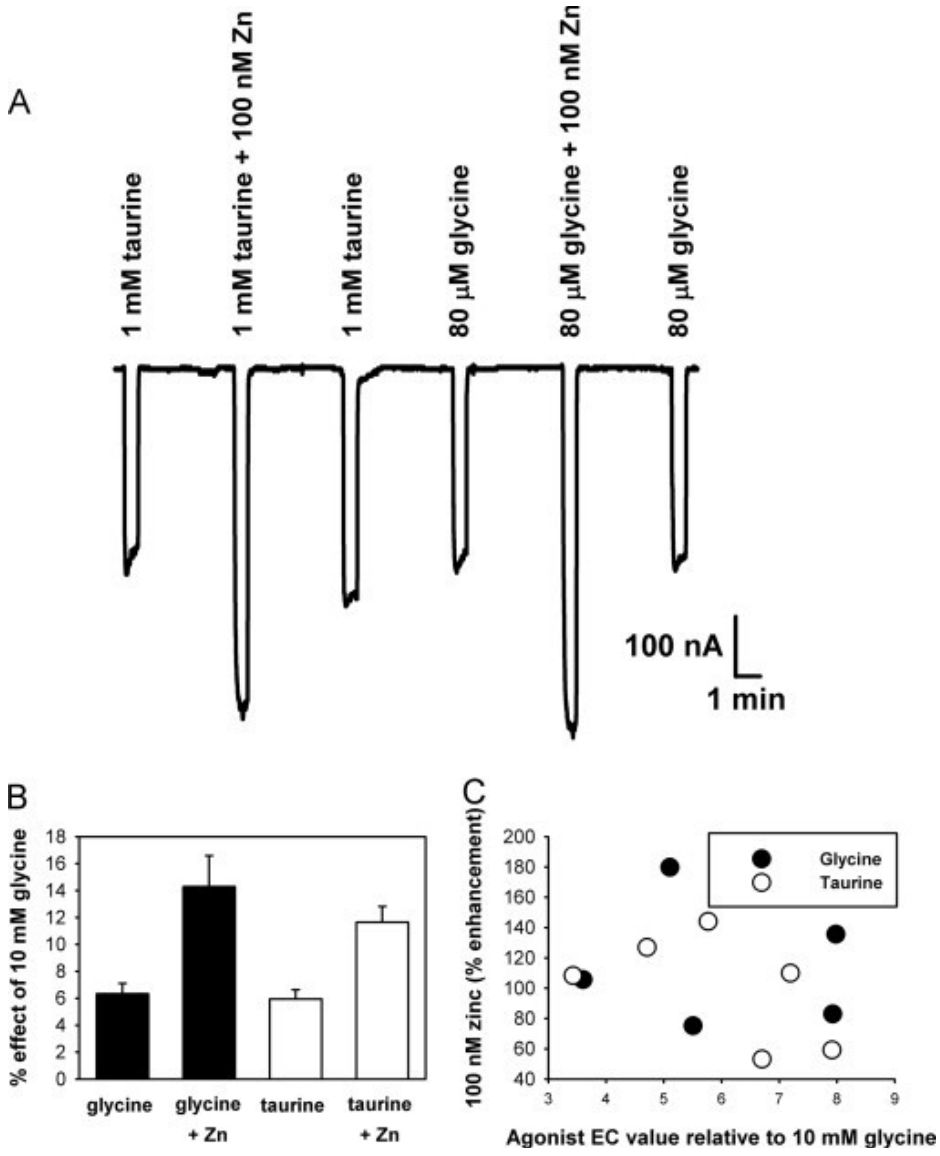


**Figure 3.2: Zinc enhancement of equi-effective glycine and taurine currents**

Zinc potentiates currents induced by low concentrations of glycine and taurine eliciting the same absolute currents, to the same degree. (A) Sample tracing showing that concentrations of glycine and taurine producing similar absolute currents result in similar degrees of enhancement by zinc. (B) Summary of the potentiating effects of 5% maximal glycine- and taurine-evoked currents by 100 nM zinc. (C) Graph showing the percentage of current enhancement of glycine and taurine currents by 100 nM zinc for individual oocytes plotted against their EC values relative to maximal glycine. Data shown for 6 oocytes.

### 3.3.3 – Ethanol enhancement of low glycine and taurine currents

In order to determine if the effects seen in my previous experiments were unique to zinc, I repeated that experimental protocol using another allosteric modulator, ethanol, in place of zinc. Co-application of 200 mM ethanol had a significant potentiating effect on glycine- and taurine-mediated currents [$F(1,19) = 123.22$, $p < 0.001$]. However, there was no difference seen in the degree of potentiation between glycine and taurine [$F(1,19) = 0.001$, $p > 0.97$] (Fig. 3.5A), despite the significant difference [$T(8) = 3.2$, $p < 0.014$] in their respective agonist EC values (Fig. 3.5B, inset). All assays in this experiment were performed using a single preparation of MBS so that background contaminating levels of zinc would be constant throughout the experiment. Data shown for individual oocytes (Fig. 3.5B) show less variability than seen previously when the concentration of contaminating zinc was not controlled (Fig. 3.2B).

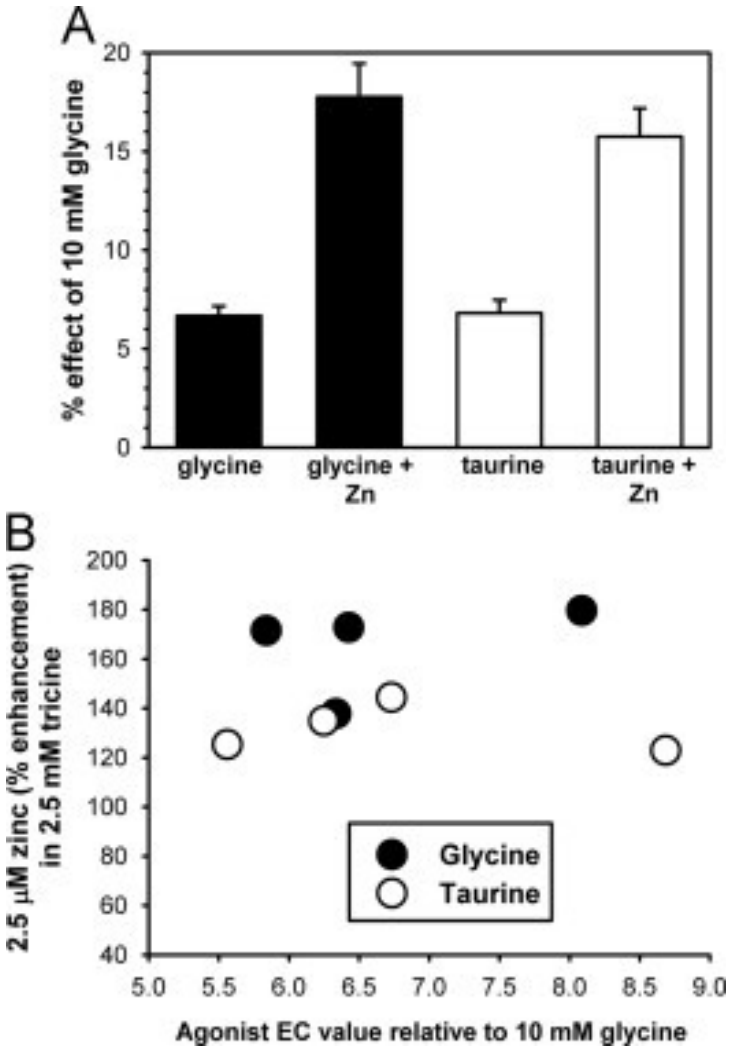


**Figure 3.3: Effects of tricine on zinc enhancement of low glycine and taurine currents**

Enhancement of GlyR function depends on the current produced rather than the agonist EC value. (A) The degree of potentiation by 2.5 μM zinc on currents elicited by low concentrations of glycine and taurine is the same for both agonists at concentrations producing the same absolute currents. (B) Percent enhancement of glycine- and taurine-mediated currents plotted against agonist EC value relative to a saturating concentration of glycine. Applications were carried out in solutions containing 2.5mM tricine. Data are shown as mean ± S.E.M. of 4 oocytes.

## 3.4 – Discussion

The GlyR is responsible for mediating much of the neuronal inhibition in the brainstem and spinal cord, although these receptors are also found in a number of higher brain regions (Baer et al 2009; Jonsson et al 2012, 2009; Lynch 2004). A variety of structurally-diverse allosteric modulators are known to affect GlyR function including divalent cations, alcohols, anesthetics and numerous drugs of abuse (Beckstead et al 2000; Harvey et al 1999; Kirson et al 2013, 2012; Mihic et al 1997). Taurine is the second most abundant amino acid in the brain and may play a role in many brain regions as an agonist acting at the GlyR (Albrecht et al 2005, Mori et al 2002). Although allosteric modulation of glycine-activated receptors has been quite extensively studied, not as much is known about modulation of GlyR activated by taurine.

Depending on whether they are positive or negative allosteric modulators at the GlyR, compounds such as ethanol, inhalants and zinc either leftshift or rightshift glycine concentration-response curves, but have minimal to no effects when co-applied with saturating concentrations of glycine (Beckstead et al., 2000; Miller et al., 2005; Welsh et al., 2010). The greatest percent enhancing or inhibiting effects of these agents are thus seen when low concentrations of agonists are tested and this is likely due to an enhancement of glycine affinity for its receptors. However, these allosteric modulators have minimal effects when applied with saturating concentrations of glycine (Fig. 3.4B). In contrast, Kirson et al. (2012) showed marked enhancement by ethanol, volatile anesthetics and inhaled drugs of abuse when co-applied with maximally-effective

concentrations of taurine. The same phenomenon was later shown using 100 nM zinc (Kirson et al. 2013). The effects of these agents at saturating concentrations of taurine cannot be due to their enhancement of taurine binding but must instead be due to their increasing the probability of channel opening (Po) subsequent to binding. In contrast, since a saturating concentration of glycine produces a Po of approximately 0.95 (Lape et al., 2008), there is little room for enhancement of Po by modulators. I tested whether the enhancing effect of zinc on taurine-activated GlyR would only occur through its effects on Po. If so, then it seems unlikely that the zinc percent potentiation should depend on the taurine concentration tested; i.e., I would have expected a flat line in Fig. 3.4A. Instead I observed progressively greater enhancement as the taurine concentration decreased, until it reached approximately 200% at low taurine concentrations (Fig. 3.4A). This was markedly greater than the ~120% maximal enhancement seen when low concentrations of glycine were tested (Fig. 3.4B). These studies did not, however, address allosteric enhancement of currents induced by low concentrations of agonist. I sought to determine whether the enhancement by allosteric modulators seen at GlyRs activated by low concentrations of taurine is also due solely to increasing Po.

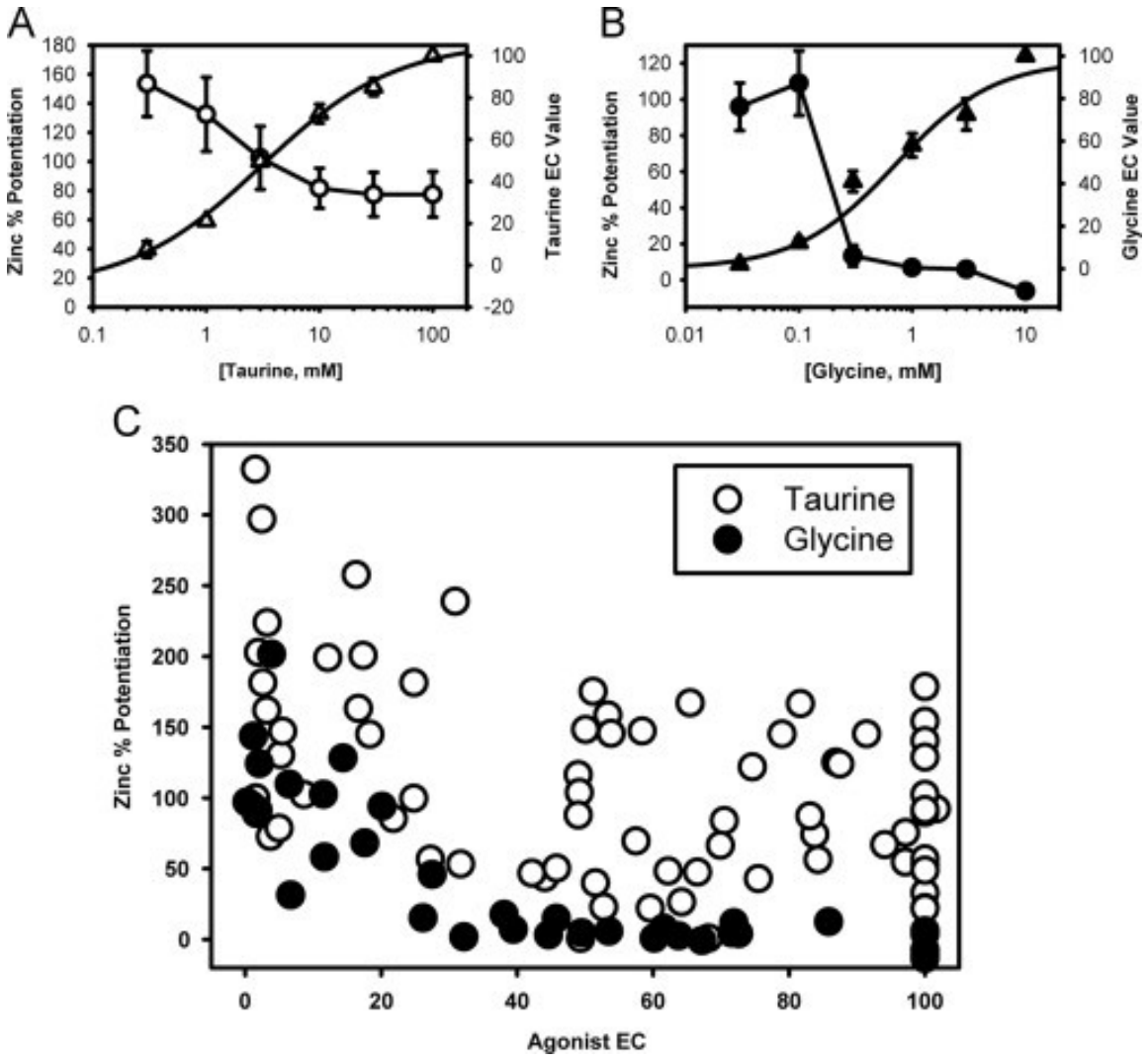


**Figure 3.4: Concentration dependence of zinc effects on glycine and taurine currents**

Zinc enhances taurine- and glycine-mediated GlyR currents in a concentration-dependent manner. (A) Taurine and (B) glycine concentration response curves and percent enhancement produced by 2.5 μM zinc at each concentration. (C) Zinc enhancement of GlyR function plotted against the respective glycine and taurine EC values. All tests were carried out in solutions containing 2.5mM tricine.

For this purpose, I began my work by comparing how 100 nM zinc affected the GlyRs response to low concentrations of glycine vs. taurine. In order to properly compare effects on receptor activity, I determined the concentrations of glycine and taurine that produced the same absolute current. These concentrations corresponded to drastically different relative EC values for these agonists (Fig. 3.1). I expected that the degree of zinc potentiation would be lower for taurine than glycine as I was much higher on the taurine concentration response curve. My data, however, showed the same degree of enhancement for both agonists (Fig. 3.2A), indicating that zinc potentiation was dependent on the amount of current produced.

Closer examination of the individual data points, revealed a great deal of variation. A recent study, however, had shown that there are nanomolar concentrations of contaminating zinc present in our buffers which would affect GlyR function. Furthermore, these concentrations vary from one of solution to the next (Cornelison et al., 2014). Based on this information, I thought it possible that the variation among my individual data points (Fig. 3.2B) may be due to varying levels of contaminating zinc present in different batches of my solutions. In this case, depending on the degree of variation in zinc contamination, my results might not have been reliable.

In order to mitigate this problem and increase accuracy, I repeated my experiments using a higher concentration of zinc (2.5 μM) and added 2.5 mM tricine to all of my buffers to chelate out background levels of zinc. My results showed no difference in the degree of potentiation of glycine- and taurine-evoked currents (Fig. 3.3),

which was consistent with the findings of my previous experiment (Fig. 3.2A). I saw similar results when with 200 mM ethanol (Fig. 3.5), indicating that the mechanism of allosteric enhancement of taurine currents is not unique to zinc.

Zinc was able to enhance currents elicited by maximally-effective concentrations of taurine by increasing Po (Kirson et al 2013). To determine if potentiation of taurine currents was solely attributable to increasing Po I measured the percent enhancement of taurine-activated currents across a wide range of taurine concentrations. If Po were the only determining factor, one would expect the percent current enhancement to be the same for all concentrations of taurine. My data showed that current enhancement at low concentrations of taurine was significantly higher than at high concentrations (Fig. 3.4). This suggests that allosteric modulators such as zinc also affect taurine affinity at these lower concentrations, by enhancing taurine binding rates or antagonizing taurine unbinding.

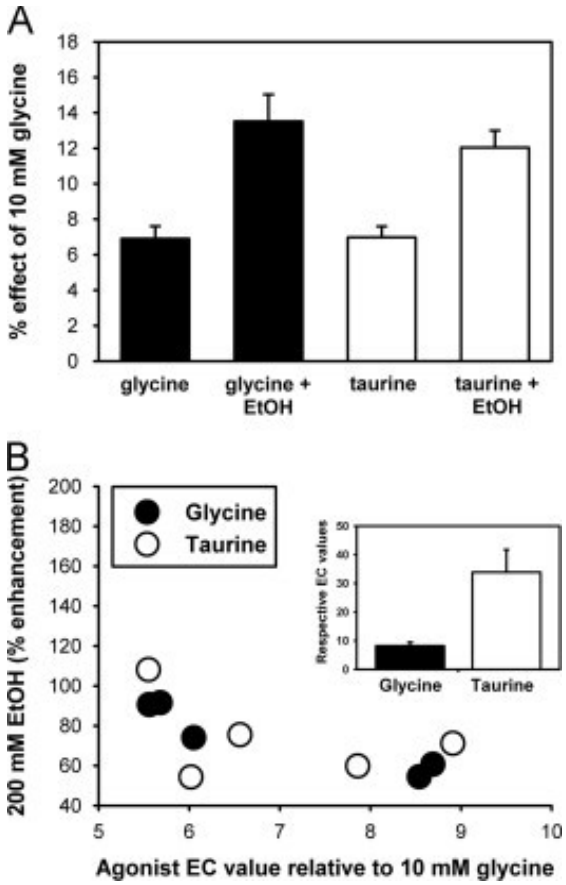


**Figure 3.5: Enhancement of matching glycine and taurine currents by 200 mM ethanol.**

At low agonist concentrations, ethanol potentiation of glycine- and taurine-evoked currents equal to 5-10% of maximal current equivalent and dependent on the absolute current produced. (A) Summary of the potentiating effects of 5-10% maximal glycine- and taurine-evoked currents by 200 mM EtOH. (B) Graph showing the percentage of current enhancement of glycine and taurine currents by 200 mM ethanol for individual oocytes plotted against their EC values relative to maximal glycine. Data are shown for 5 oocytes.

# Chapter 4: Mutation-induced changes in agonist efficacy alter allosteric modulation of the glycine receptor

## 4.1 – Introduction

The glycine receptor (GlyR) is the primary mediator of inhibitory neurotransmission in the brainstem and spinal cord and has also been found in higher brain regions such as the hippocampus, prefrontal cortex and nucleus accumbens (Baer et al 2009; Jonsson et al 2012, 2009; Lynch 2004). Four alpha and one beta subunit have been discovered of which, all but α4 can be found in humans. GlyRs are expressed endogenously as homopentamers composed solely of alpha subunits or as heteropentamers with a stoichiometry of 2 α : 3 β (Betz et al 1993; Bowery et al 2006; Lynch 2004) or 3 α : 2 β (Grudzinska et al., 2005) arranged around a central anion-conducting channel. Taurine is a partial agonist exhibiting approximately 5% the efficacy of glycine at WT (WT) GlyR (Lape et al. 2008). Although glycine is the prototypical agonist *in vivo*, evidence exists supporting the role of taurine as an important GlyR agonist in numerous brain regions (Albrecht and Schousboe 2005). This is supported by the work of Mori et al. (2002) who showed that a taurine uptake inhibitor induced a strychnine-sensitive chloride current in hippocampal organotypic slice cultures.

GlyR function is modulated by a variety of endogenous compounds such as zinc, endocannabinoids and neuroactive steroids (Laube et al., 2000; Lynch, 2004; Yevenes, et.al., 2011) as well as numerous addictive drugs including alcohols, anesthetics and inhalants (Beckstead et al., 2000; Harvey et al., 1999; Kirson et al., 2013, 2012; Mascia et

al. 1996; Mihic et al., 1997). The divalent cation zinc is present both *in vivo* and as a contaminant in buffers at concentrations sufficient to modulate GlyR function (Frederickson et al, 2006a, 2006b; Hirzel et al, 2006; Cornelison and Mihic, 2014). This biphasic modulator interacts with some other modulators, such as ethanol, to affect receptor activity (Bloomenthal et. al., 1994; Cornelison et al., 2017; McCracken et al., 2013, 2010). The tryptophan to serine mutation at residue 170 (W170S) of the α1 GlyR leads to one form of human hyperekplexia (Zhou et al., 2013). This mutation yields receptors insensitive to enhancing concentrations of zinc (<10uM), thus allowing for the study of allosteric modulation of GlyR without the confounding effects of zinc enhancement.

GlyRs have been implicated in alcohol-related behaviors. McCracken et al. (2013) found that C57BL/6 mice expressing the D80A mutation in the α1 subunit of the GlyR showed a reduction in ethanol consumption and preference. This is consistent with earlier studies showing that the GlyR mediates behavioral actions of drugs of abuse (Li et al., 2012; Molander et. al, 2005; Ye et. al, 2001) and further highlights its potential as a target for the treatment of addiction. Two putative alcohol binding sites have been identified on the GlyR. S267 and A288 in the transmembrane domain of α1 GlyR form part of a binding pocket that is critical for the potentiating actions of alcohols and anesthetics (McCracken et al., 2016; Mihic et al., 1997) The potentiating effects of ethanol on glycine-evoked currents were also attenuated in the murine α1 A52S *spasmodic* mutant (Mascia et al., 1996). It has since been proposed that the A52-

containing Loop 2 region of the α1 GlyR forms a second binding site for ethanol (Naito et al., 2014; Perkins et al., 2008, 2012). All of these studies, however, have focused on glycine-activated GlyR but, to our knowledge, no one has characterized the effects of taurine on GlyR Loop 2 mutants.

In earlier experiments, I found that the degree of potentiation of WT homomeric α1 GlyR currents produced by ethanol is greater, across all agonist concentrations, for taurine than glycine. I believe that this effect may be due to taurine having a lower efficacy than glycine. I hypothesized that mutation-induced changes in allosteric modulation may be due to changes in agonist efficacy produced by these mutations. To explore this possibility, I chose to examine GlyR mutants that displayed altered responses to known GlyR modulators.

Previous studies by (Wallner et al. 2006, 2003) claimed that $GABA_A$ receptors containing the δ subunit are sensitive to very low concentrations of ethanol. Based on this information, Naito et al. (2015, 2014) generated a series of mutations in the Loop 2 of the glycine receptor based on the corresponding sequence in the $GABA_A$ δ subunit that they named, Ultra-Sensitive to Ethanol Receptors (USERs). They reported astonishingly low ethanol sensitivities at these receptors, making them attractive models with which to test our theory.

I attempted to characterize the ethanol sensitivity of USERs 1-3 as well as a series of partial USER mutants generated in the lab, that contained some, but not all, of the mutated residues found in USER3, with the hope of identifying the contributions of

specific residues to this phenotype. I also examined the actions of taurine and ethanol on A52S and W170S mutant receptors, which display decreased and increased taurine efficacy, respectively, allowing me to further explore the link between agonist efficacy and allosteric modulation.

## 4.2 – Materials and Methods

### 4.2.1 - Generation of Point Mutations

The α1 A52S and W170S point mutations were generated via site-directed mutagenesis using the methods described in Chapter 2. The USER mutants were a gift from the Davies lab at the University of Southern California (Los Angeles, CA).

### 4.2.2 – Two-electrode voltage-clamp electrophysiology

Oocyte isolation, injection and two-electrode voltage-clamp electrophysiology experiments were completed using the methods outlined in Chapter 2 with the following modifications.

Oocytes were preincubated with modulators for 30-60s prior to co-application with either glycine or taurine. Agonist applications lasted 30s for submaximally-effective agonist concentrations and 15-30s for maximally-effective concentrations of agonists. Washout periods ranged from 3-15 minutes, depending on the concentration of agonist used, to allow for receptor resensitization. Data were acquired using a Powerlab 4/30 digitizer with LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia) and stored on a computer hard drive for later analysis.

### 4.2.3 - Data Analysis

Peak currents observed in the presence of agonist were compared with currents produced by agonist with modulator. Values are listed as the mean ± S.E.M. *t*-tests, paired t-tests, 2-way and 3-way ANOVAs were used to determine statistical significance, as indicated. Statistical testing was performed using SigmaPlot version 11.0 (Systat Software, San Jose, CA).

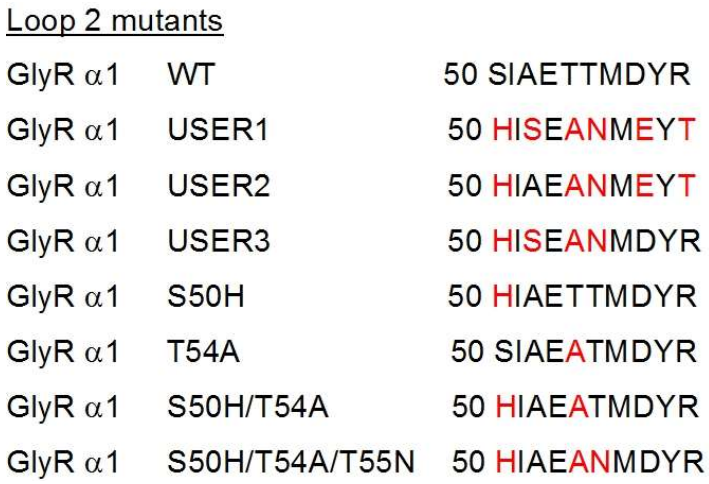


**Fig 4.1 - Sequence alignment of the α1 GlyR Loop 2 domain of wildtype, USER and partial USER mutants**

Sequence alignment of wildtype, USER and partial USER mutations in Loop2 of the extracellular domain. Mutated residues are shown in red.

## 4.3 – Results

### 4.3.1 – Effects of Loop 2 mutations on ethanol modulation of α1 glycine receptors

Perkins et al. (2009) and Naito et al. (2014) reported that replacing a region of loop 2 of the α1 GlyR and γ2 $GABA_AR$ with the equivalent amino acids found in the $GABA_AR$ δ subunit substantially increased ethanol sensitivity at these USER (ultra-low sensitivity to ethanol receptor) mutants. USERS 1, 2 and 3 contain 6, 5, and 4 mutations in Loop 2, respectively (Figure 4.1). I generated a series of mutants based off of the USER3 sequence, shown in (Figure 4.1) to determine if a subset of these residues was responsible for the enhanced ethanol sensitivity reported in the literature. Unfortunately, none of my mutants resembled USER in their responses to ethanol (Data not shown).

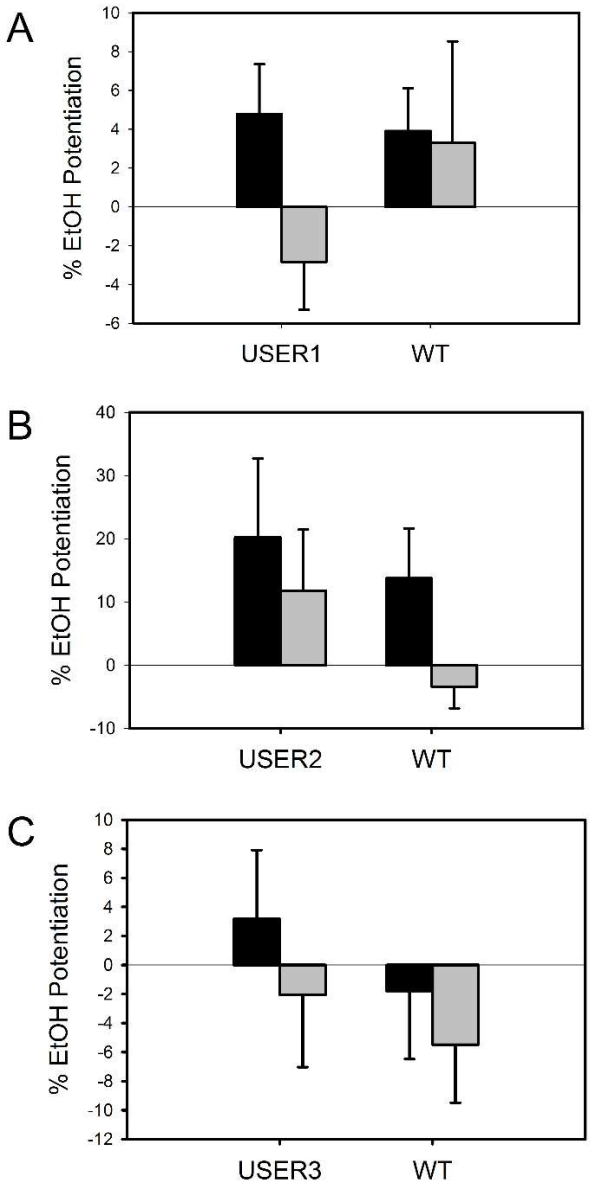


**Fig 4.2 - Effects of low concentrations of ethanol on $EC_2$ glycine-activated USER vs. wildtype GlyRs**

Summary graphs showing the degree of potentiation by 500 μM (grey bars) or 1 mM (black bars) ethanol of currents produced by low ($EC_2$) glycine. (A-C) There is no significant difference in the degree of enhancement between wildtype and USER glycine receptors at either concentration. Data are shown as mean ± S.E.M. of 6-9 oocytes.

I next attempted to confirm the original USER data by testing the effects of 500 μM and 1 mM EtOH on $EC_2$ glycine-activated receptors. As shown in Figure 4.2, none of the USER mutants differed significantly from WT in their response to low concentrations of ethanol. Despite my failure to reaffirm the nature of the purported ultra-sensitive mutants, I wondered how they would respond to taurine. I measured the effects of maximally-effective concentrations of taurine and glycine at the three USER mutants. I found no significant difference in the relative efficacy of taurine at USER1 [$t(12) = 1.01$, $p > 0.33$] and USER3 [$t(11) = 0.17$, $p > 0.86$] but saw a reduction in average taurine efficacy at USER2 (Figure 4.3). Next, I coapplied 200 mM EtOH with max taurine to determine if there was a change in ethanol modulation. Again, USERs 1 [$t(12) = 1.71$, $p > 0.11$] and 3 [$t(11) = 0.55$, $p > 0.59$] did not differ significantly from WT, but USER2 displayed a marked increase in EtOH potentiation of currents elicited by maximally-effective taurine concentrations [$t(18) = 3.18$, $p < 0.006$] (Figure 4.4).

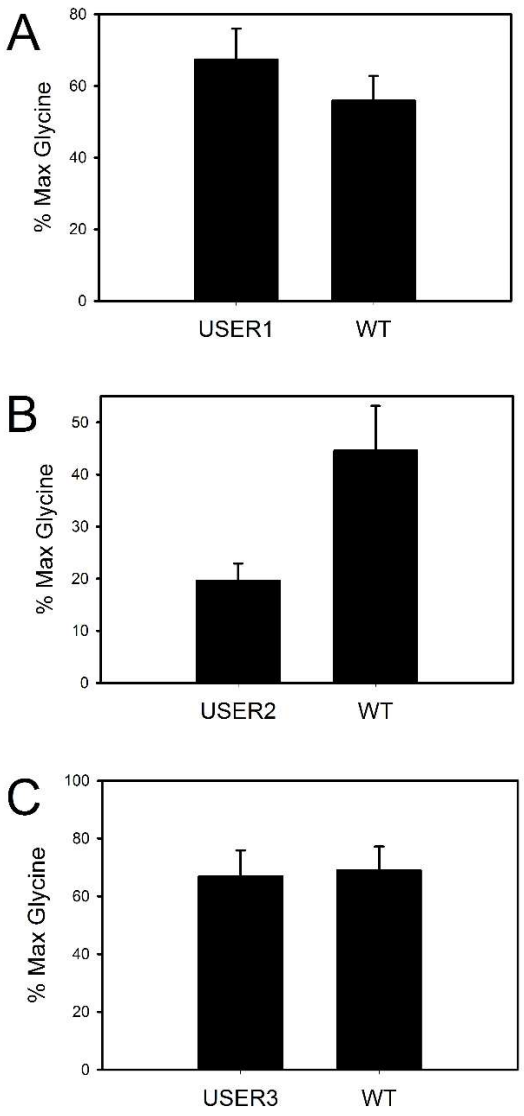


**Fig 4.3 - Relative efficacy of taurine currents at wildtype and USER α1 glycine receptors**

Graphs showing the average peak currents produced by maximally effective concentrations of taurine relative to glycine at USER and WT GlyRs. The relative efficacy of taurine at (A) USER1 and (B) USER3 GlyRs does not differ significantly from WT. (B) Taurine has decreased efficacy at USER2. All data are normalized to currents produced by 10mM glycine. Data are shown as mean ± S.E.M. of 5-12 oocytes.

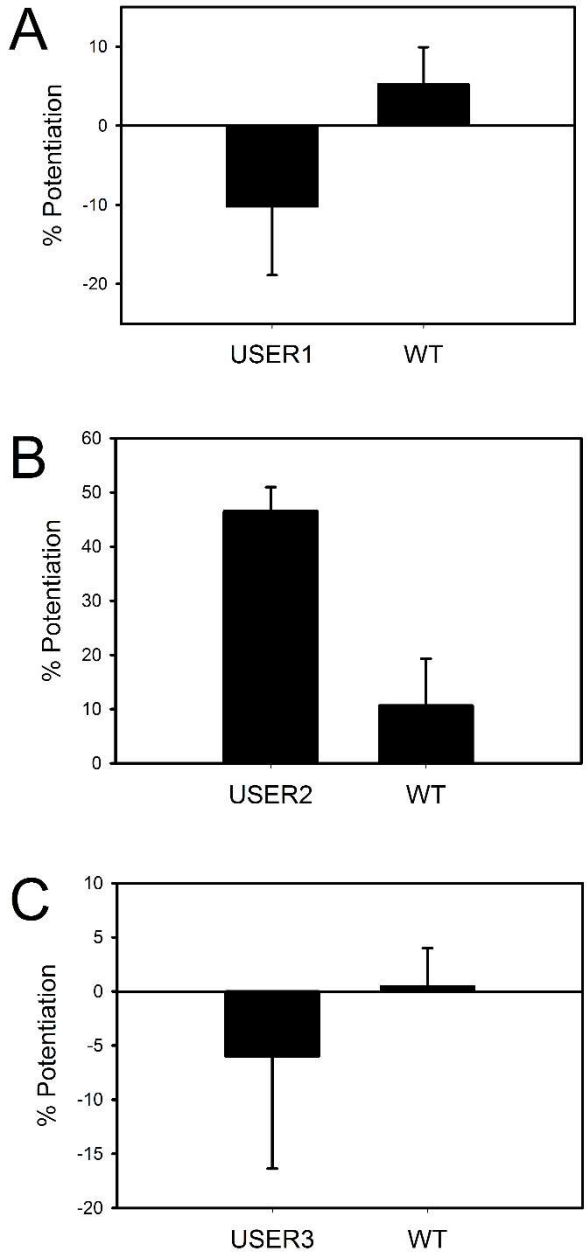


**Fig 4.4 - Ethanol potentiation of max taurine currents at USERs**

Summary graphs showing the percent potentiation of saturating taurine currents after co-application with 200 mM ethanol. There is no significant difference in the degree of ethanol potentiation of maximally effective taurine currents between (A) USER1 or (C) USER3 and WT. (B) Ethanol potentiation of saturating taurine currents is significantly increased at USER2 GlyRs. Data are shown as mean ± S.E.M. of 5-12 oocytes.

### 4.3.2 – Characterization of taurine-activated α1 A52S GlyR enhancement by zinc and ethanol

The only difference between GlyR USER1 and USER2 is the A52S mutation found in USER1. Mascia et al. (1996) previously found that the α1 A52S mutation resulted in a decreased sensitivity to glycine and reduced potentiation of glycine-evoked currents by ethanol. These data suggest that the residue at position 52 has an important role mediating the effects of ethanol at glycine-activated GlyRs, however, the effects of the A52S mutation on taurine-evoked currents was still unknown.

I characterized the actions of taurine at α1 glycine receptors compared to WT. Concentration response curves for both glycine and taurine were constructed. The agonist concentration-response curve was right-shifted in the mutant when glycine was the agonist, which agrees with the findings of Mascia et al. (1996). A two-way ANOVA showed a significant effect of receptor when activated by glycine [$F(1,95) = 6.46$, $p < 0.014$]. However, no significant effect of receptor was seen between WT and A52S in response to taurine [$F(1,89) = 0.72$, $p > 0.39$] (Fig. 4.5).

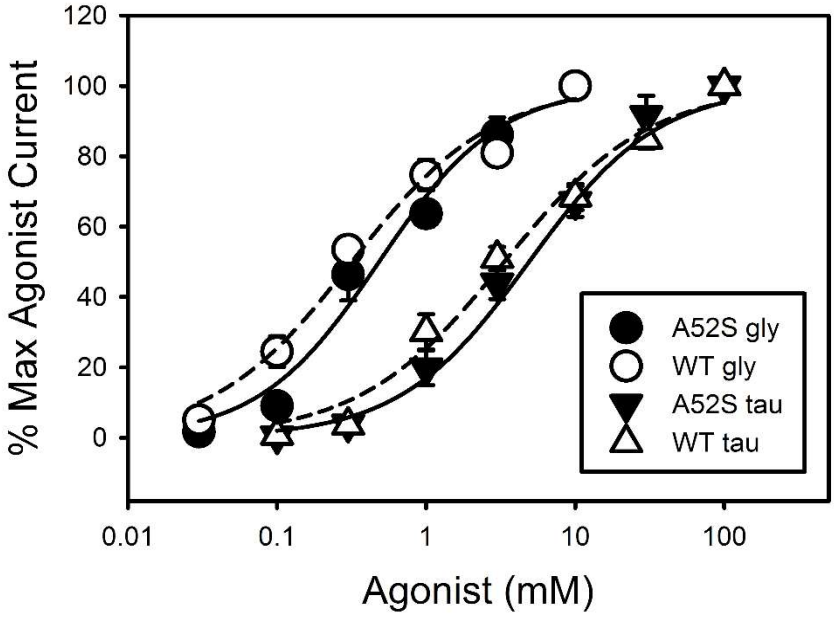


**Fig 4.5 - Agonist concentration response curves for wildtype and A52S α1 glycine receptors**

Concentration response curves for glycine (circles) and taurine (triangles) in *Xenopus laevis* oocytes expressing either homomeric $\alpha 1^{A52S}$ or WT GlyRs. Values are normalized to the maximal currents elicited by each agonist. The A52S mutant is less sensitive to glycine than WT. There is no significant effect of receptor when taurine is the agonist. Data are shown as mean ± S.E.M. of 4-9 oocytes.

The enhancing effects of ethanol on currents produced by submaximal concentrations of glycine and taurine were next tested in WT and A52S GlyR. In each oocyte, concentrations of glycine and taurine were determined that produced currents corresponding to 5-10% of the maximal response ($EC_{5-10}$) to each agonist. These were then co-applied with 50, 100 or 200 mM ethanol. Co-application with ethanol increased the degree of potentiation of taurine-mediated currents in the A52S mutant by ethanol when compared to WT [$F(1,65) = 5.692$, $p = 0.020$] (Fig 4.6A). However, contrary to what was reported by Mascia et al. (1996), no significant difference in the degree of ethanol potentiation of currents produced by glycine was seen [$F(1,66) = 1.18$, $p = 0.28$] (Fig. 4.6B).

Taurine efficacy relative to glycine was determined by comparing current amplitudes produced by saturating concentrations of both agonists. Figure 4.7A shows that 100 mM taurine has significantly lower efficacy at A52S than WT $\alpha$1 GlyR, relative to the currents elicited by 10 mM glycine in those receptors [$t(8) = 3.98$, $p < 0.005$].

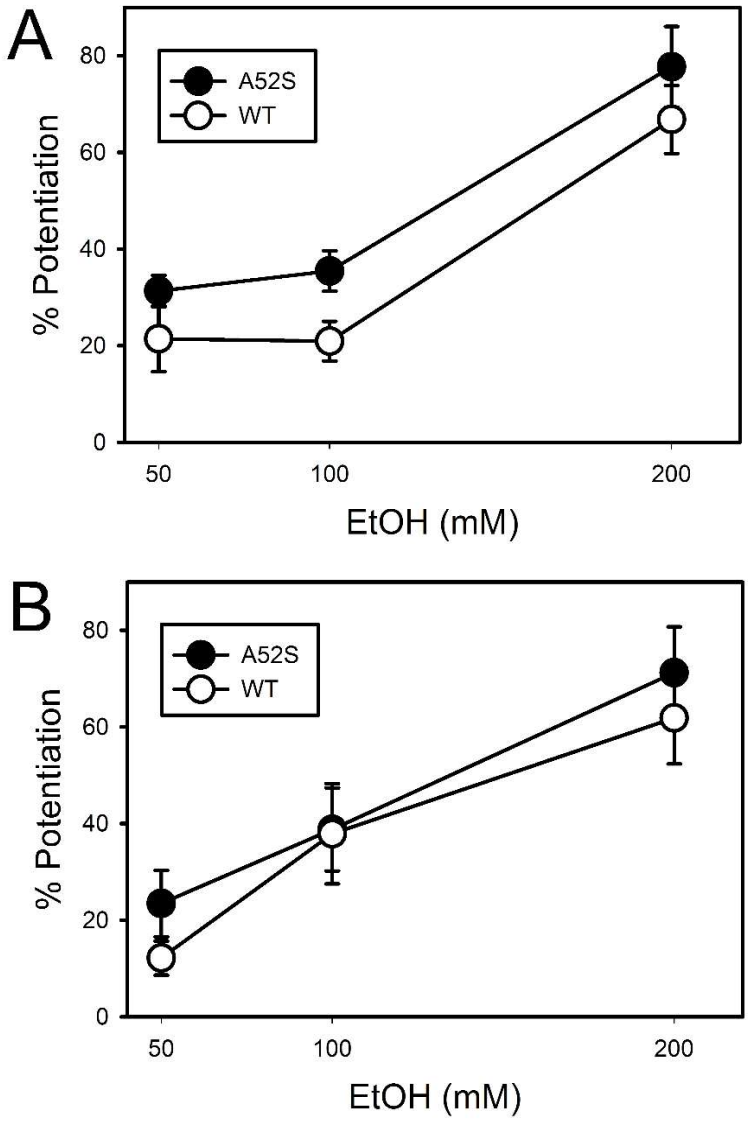


**Fig 4.6 - Ethanol modulation of low glycine- and taurine-evoked currents at wildtype and A52S**

Effects of enhancing concentrations of ethanol on WT and A52S glycine receptors activated by $EC_{5\text{-}10}$ glycine or taurine. (A) Ethanol enhances currents elicited by the partial agonist taurine to a greater degree in $\alpha 1^{A52S}$ mutant receptors than WT. (B) There was no difference in the degree of ethanol enhancement of glycine-evoked currents between WT and A52S. Data are presented as mean ± S.E.M. for 7-10 oocytes.

Previously I hypothesized that the greater degree of allosteric modulation seen in GlyRs that are activated by taurine compared to glycine may be related to the significantly lower efficacy of taurine at WT receptors (Farley and Mihic, 2015). Maximally-effective taurine-activated currents are potentiated by ethanol and zinc which must be due to an increase in the probability of channel opening (Po).

Since taurine has reduced efficacy at A52S $\alpha$1 GlyR I tested whether there was a corresponding increase in the degree of ethanol potentiation. Peak currents elicited by 100 mM taurine were compared to those produced by co-application of taurine with ethanol. Ethanol, at a concentration of 200 mM enhanced taurine currents to a greater extent in A52S than in WT $\alpha$1 GlyR [$t(8) = 2.73$, $p < 0.03$] (Fig. 4.7B).

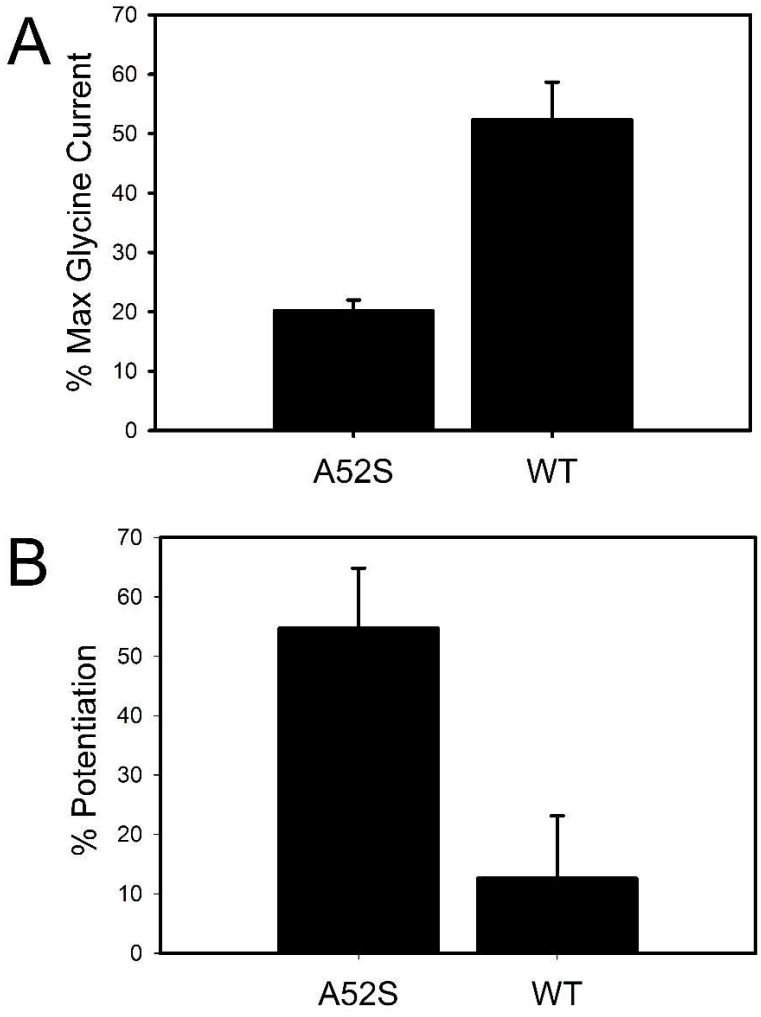


**Fig 4.7 - Relative efficacy of taurine at wildtype and A52S GlyRs**

Peak currents produced by saturating concentrations of glycine and taurine. (A) Taurine has lower efficacy relative to glycine at $\alpha 1^{A52S}$ vs. WT GlyRs. (B) Enhancement of maximally effective taurine currents by 200mM ethanol is greater at A52S than WT. All data are normalized to currents produced by saturating glycine. Data are shown as mean ± S.E.M. of 4-6 oocytes.

Since GlyR function is affected by nanomolar, contaminating levels of zinc found in perfusion buffers (Cornelison and Mihic, 2014), some of the differences observed between WT and A52S GlyR might be due, in part, to differences in zinc sensitivity between these receptors. To determine if this was the case, the degree of zinc modulation between WT and mutant A52S GlyR was compared at low and maximally-effective concentrations of taurine (Figure 4.8). First, the concentration of taurine that produced $EC_{4\text{-}10}$ currents was determined and then the degree of modulation produced by co-application of taurine with 0.1 and 1 μM zinc, concentrations of which potentiate currents at WT receptors (Figure 4.8). Similarly, I measured the effects of 100 μM zinc, which inhibits GlyR currents (Harvey et al., 1999; Laube et al. 2000). I then repeated these experiments using 100 mM taurine (Figure 4.8). A two-way ANOVA showed no significant effect of receptor at either $EC_{4\text{-}10}$ [$F(1,40) = 1.51$, $p > 0.22$] or saturating concentrations of taurine [$F(1,35) = 0.92$, $p > 0.34$] (Fig 4.8).

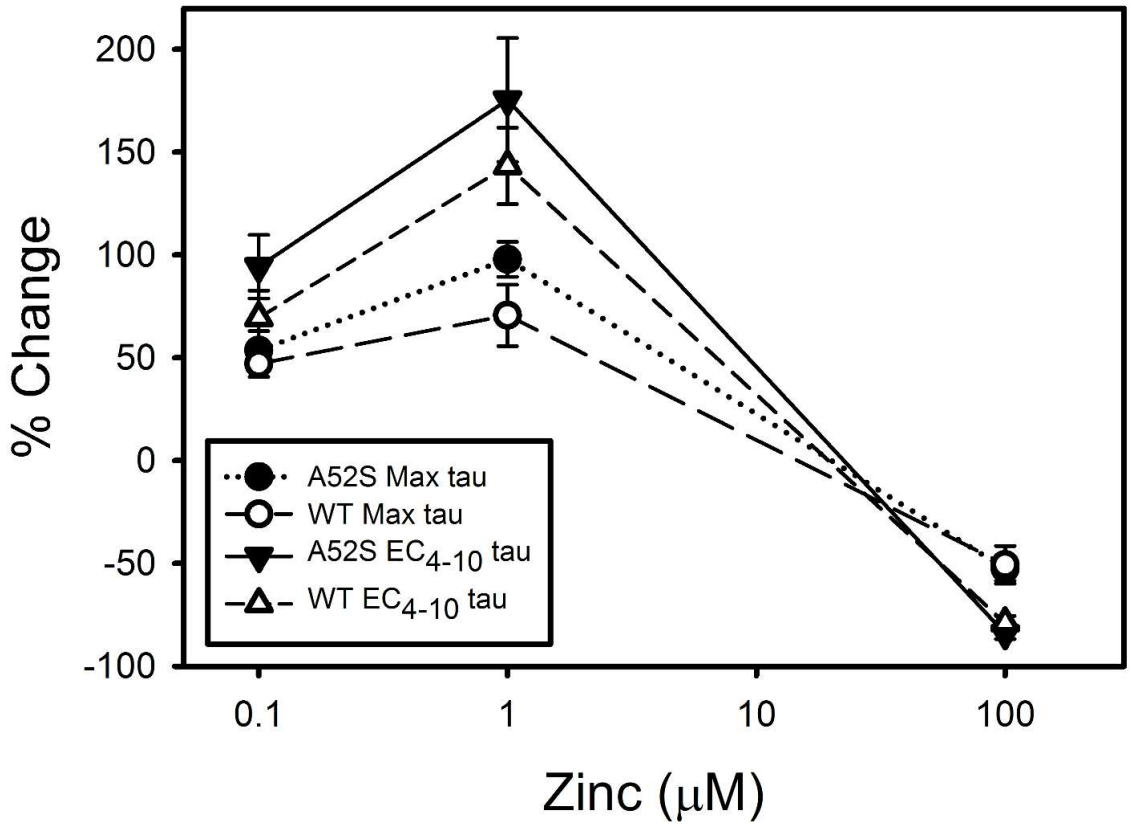


**Fig 4.8 - Effects of zinc at taurine-activated wildtype and A52S receptors**

Effects of 100 nM, 1 μM and 100 μM $ZnCl_2$ on WT and $\alpha1^{A52S}$ GlyRs activated by low (triangles) and high (circles) concentrations of taurine. Zinc modulates currents elicited by $EC_{4-10}$ and maximally effective concentrations of taurine to the same extent in A52S and WT receptors. Data are shown as mean ± S.E.M. of 5-8 oocytes.

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### 4.3.3 – Effects of zinc and ethanol on taurine-evoked currents at W170S glycine receptors

Cornelison et al. (2017) demonstrated that the W170S α1 GlyR human hyperekplexia mutant, which is insensitive to zinc potentiation, can be used to study ethanol modulation of glycine-activated GlyRs without the added complication of zinc modulation. I characterized the effects of taurine at this receptor. Taurine concentration-response curves for W170S and WT GlyR were first generated (Figure 4.9). Although the W170S curve appears slightly right-shifted, a two-way ANOVA showed no significant effect of receptor [$F(1,64) = 0.33$, $p > 0.56$]. Interestingly, as seen in Figure 4.10A, taurine has much higher efficacy at the W170S α1 GlyR compared to WT receptors.

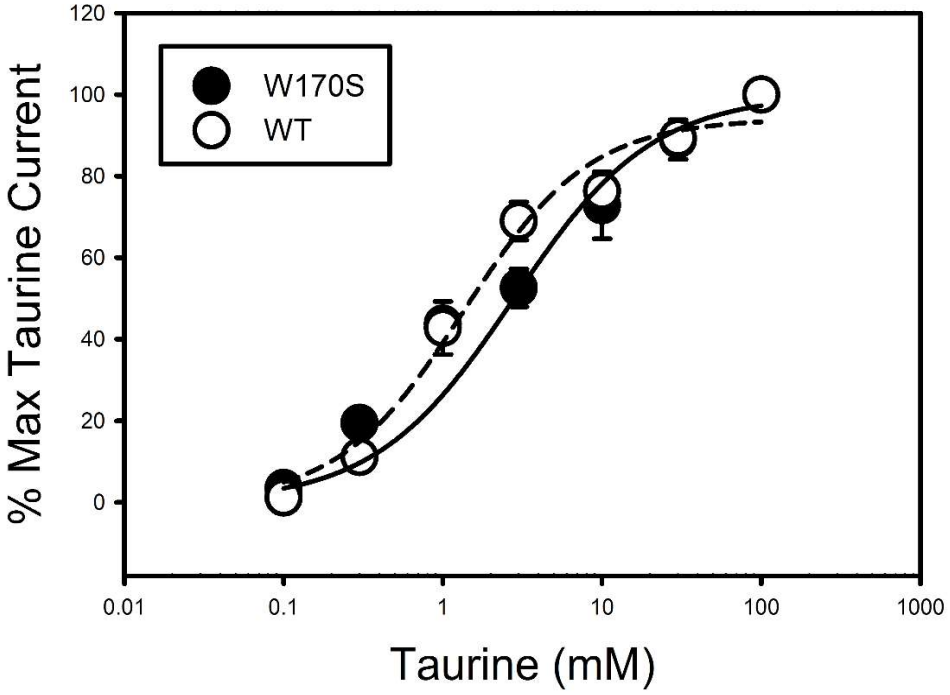


**Fig 4.9 - Taurine concentration response curves for wildtype and W170S α1 glycine receptors**

Concentration response curves for whole-cell taurine currents in oocytes expressing homomeric $\alpha1^{W170S}$ or WT GlyRs. There is no significant effect of receptor between W170S and WT. Values are expressed as a percentage of the maximal taurine current in each oocyte. Data are shown as mean ± S.E.M. of 3-5 oocytes.

Due to its high efficacy, the probability of channel opening for WT GlyR at saturating glycine concentrations is near 1. As a result, ethanol has minimal effects at receptors activated by high concentrations of glycine (Kirson et al., 2012). Since taurine has much higher efficacy relative to glycine at W170S GlyR, I postulated that maximally-effective taurine currents would display a similar absence of ethanol potentiation. Upon comparing the effects of 200mM ethanol on currents produced by 100 mM taurine, significantly greater effects were found in WT than W170S $\alpha$1 GlyR [$t(14) = 3.46$, $p < 0.005$] (Figure 4.10B).

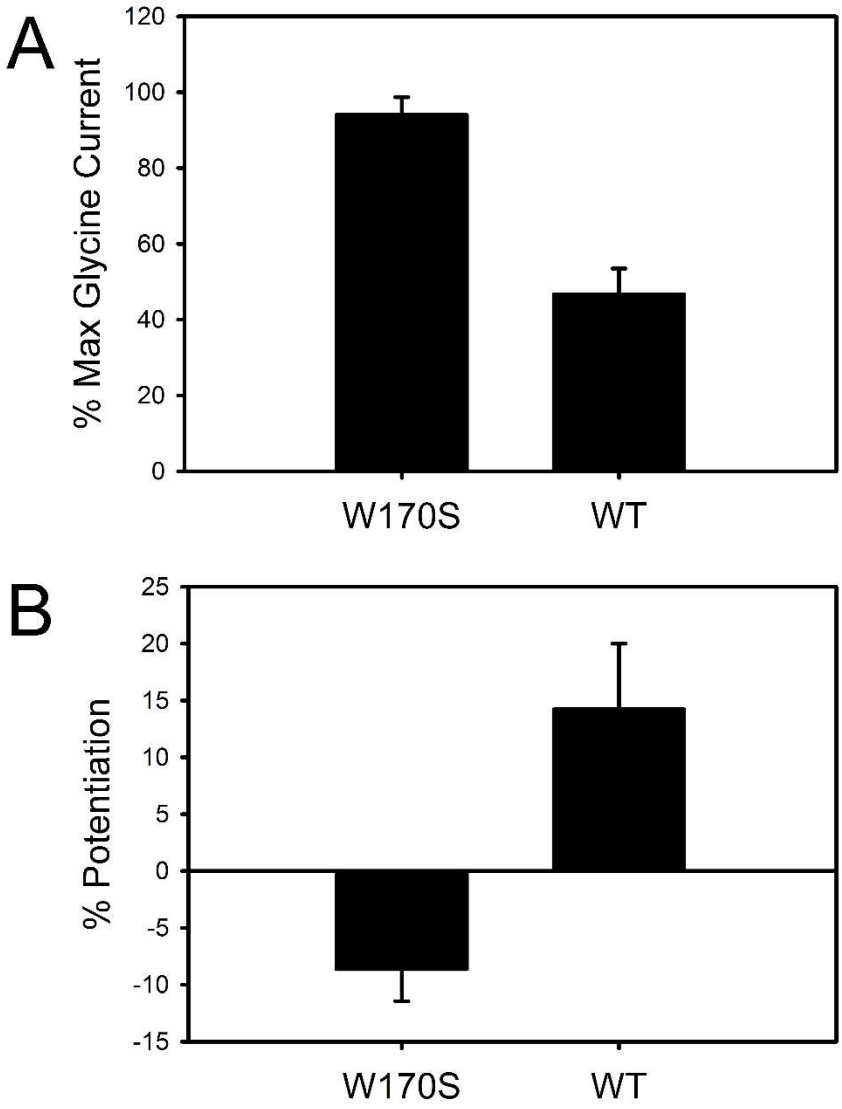


**Fig 4.10 - Relative efficacy of taurine at wildtype and W170S GlyRs**

Magnitude of 100 mM taurine currents and effects of 200mM ethanol on taurine-activated $\alpha 1^{W170S}$ and WT glycine receptors. (A) The W170S mutation drastically increases taurine efficacy and (B) abolishes ethanol potentiation of currents produced by saturating concentrations of taurine. Data are shown as mean ± S.E.M. of 8-9 oocytes.

W170S GlyRs exhibit a lower degree of ethanol enhancement of glycine currents when tested in standard MBS buffer, which contains low nanomolar concentrations of contaminating zinc. This difference, however, was eliminated in the presence of the zinc chelator, tricine (Cornelison et al. 2017). I performed similar experiments to determine whether the same phenomenon occurs when taurine is the agonist. I began by measuring the enhancement of $EC_{5\text{-}10}$ taurine currents by ethanol in our normal MBS buffer. The degree of potentiation produced by 50 mM ethanol was significantly lower in W170S than WT GlyR [$t(13) = 2.95$, $p < 0.015$] (Fig. 4.12A). I then repeated these experiments using MBS that contained 2.5 mM tricine. As seen with glycine previously (Cornelison et al., 2017), there was no statistically significant difference in ethanol modulation of currents elicited by low taurine concentrations between W170S and WT at either 50 or 200 mM ethanol concentration (Fig. 4.12B).

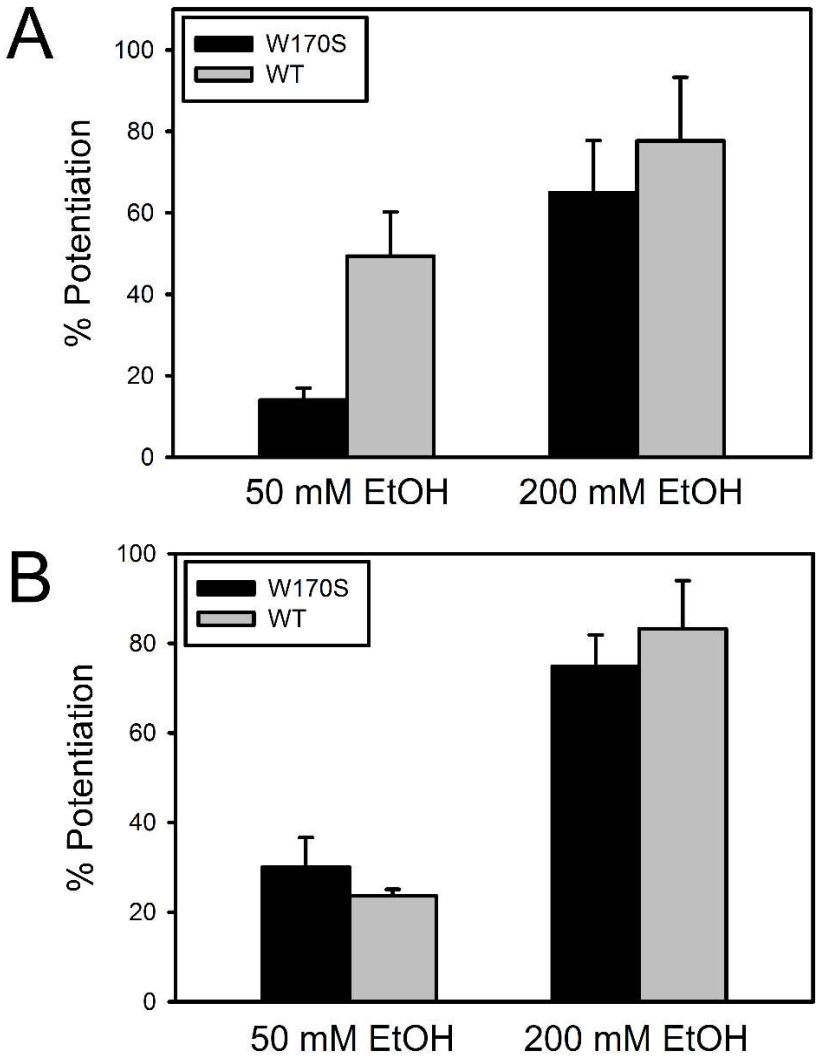


**Fig 4.11 - Effects of ethanol and tricine on low taurine-evoked currents at W170S and wildtype receptors**

Effects of zinc chelation on ethanol enhancement of W170S and WT GlyRs. (A) 50 mM ethanol enhancement of taurine currents at W170S was much lower than WT in the presence of low concentrations of contaminating zinc. Data are shown as mean ± S.E.M. of 7-8 oocytes. (B) There was no significant difference in the degree of ethanol modulation between WT and W170S in the presence of 2.5mM tricine. Data shown as mean ± S.E.M. of 5 oocytes.

## 4.4 – Discussion

A large number of allosteric modulators, such as divalent cations, neurosteroids, endocannabinoids, alcohols and anesthetics influence GlyR activity (Beckstead et al., 2000; Harvey et al., 1999; Kirson et al., 2013, 2012; Mascia et al. 1996; Mihic et al., 1997, Yevenes, et al., 2011). GlyRs are believed to play a role in the development of alcohol addiction and mediate some of the behavioral effects of ethanol (Li et al., 2012; McCracken et al, 2013; Molander et. al, 2005, 2007; Ye et. al, 2001). Thus far, most GlyR research has focused on receptors activated by glycine, however, the lower efficacy compound taurine is also believed to be an important GlyR agonist *in vivo* (Albrecht and Schousboe, 2005; Mori et al., 2002).

Positive allosteric modulators like ethanol have minimal effects at saturating concentrations of high-efficacy agonists, like glycine, because the probability of channel opening (Po) is already close to 1 when the agonist is applied alone. By comparison, the Po for WT α1 homomeric GlyRs activated by maximally-effective concentrations of taurine is only ~0.5. The abilities of modulators to increase the Po of GlyRs gated by high concentrations of low efficacy agonists explains the findings of Kirson et al. (2012, 2013) who observed significant enhancement of saturating taurine- but not glycine-evoked currents by isoflurane and zinc. I obtained similar results with ethanol and zinc at WT α1 GlyRs (Farley and Mihic, 2015) which raised the question of whether a relationship exists between the efficacy of an agonist and the degree of modulation observed when it is applied at a saturating concentration. Based on my previous results, I

hypothesized that the degree of allosteric modulation dependents on agonist efficacy, and that mutations that decrease agonist efficacy would show a corresponding increase in the magnitude of modulation. Similarly, if an agonist were to have higher efficacy at a particular mutant, this would lead to a decrease in the degree of modulation.

Loop 2 of the α1 subunit is located at the interface between the extracellular and transmembrane domains and has been hypothesized to form part of a second alcohol binding pocket (Crawford et al., 2007; Naito et al., 2014; Perkins et al., 2008, 2009, 2012). Perkins et al. (2009, 2012) generated a series of mutants in loop 2 of the α1 GlyR by mutating non-conserved residues to those present in the $GABA_A$ δ subunit (Fig. 4.1). They reported a significant decrease in the threshold ethanol sensitivity of these USER (ultra-low sensitivity to ethanol) mutants when activated by glycine. USER3 contains fewer mutations than USERs 1 and 2 with only 4 residues that differ from WT. I tested a series of mutants that contained 1-3 of the mutations found in USER3 to see if these were sufficient to confer enhanced ethanol sensitivity (Figure 4.1). The A52S mutation is known to decrease ethanol potentiation of α1 GlyR currents and was therefore excluded. Unfortunately, none of my partial USER mutants displayed an enhanced response to ethanol when compared to WT (data not shown).

Troubled by these results, I attempted to verify the ultra-sensitive phenotype of the USER mutants by repeating some of the original experiments, to the best of my ability. I tested the effects of two low ethanol concentrations that were reported to have particularly robust effects on currents produced by $EC_2$ glycine (Naito et al. 2014). As

seen in Figure 4.2, however, I was unable to substantiate these claims. In my hands, none of these USER mutants differed from WT in their response to 500 μM and 1 mM EtOH.

In spite of these findings, I thought it prudent to characterize the effects of taurine at these receptors. I tested the effects of maximally effective concentrations of taurine and glycine on oocytes expressing USERs 1-3 or WT GlyRs as well as the effects of 200mM EtOH on these receptors when activated by saturating taurine. I found no significant difference between USER1 or USER3 and WT in either experiment, however, USER2 showed a striking decrease in response to taurine relative to glycine, indicating that taurine has reduced efficacy (Figure 4.43). Furthermore, ethanol potentiation of max taurine currents in USER2 was significantly higher than the degree of potentiation observed in WT (Figure 4.4). Given that the only difference between USERs 1 and 2 is A52S, these data reinforce the idea that the residue at position 52 of the α1 subunit plays an important role in determining how glycine receptors respond to ethanol when activated by taurine. It also provides further evidence of an inverse relationship between agonist efficacy and the degree of allosteric modulation at glycine receptors. However, one curious finding is that the A52S mutation made in WT receptors decreases taurine relative efficacy while the same mutation in USER2 to make USER 1 increases taurine's efficacy relative to glycine.

Mascia et al. (1996) demonstrated that the murine *spasmodic* mutant A52S residue, located in Loop 2, decreases ethanol sensitivity in α1 homomeric GlyRs activated by glycine. However, allosteric modulation of A52S GlyRs when gated by

taurine had not yet been investigated. I expanded on the work of Mascia and colleagues to better characterize the actions of glycine, taurine and ethanol at these receptors.

In my hands, the A52S mutant showed a small decrease in glycine sensitivity relative to WT GlyR, as evidenced by the right-ward shift in the concentration response curve for A52S (Figure 4.5). This is in agreement with Mascia et al. (1996). The taurine concentration-response curves for the A52S α1 GlyR also appeared to be right-shifted to a similar degree compared to WT, but this was not statistically significant. I next compared the degree of ethanol potentiation of currents elicited by $EC_{5\text{-}10}$ glycine and taurine at A52S and WT GlyR and found that the degree of potentiation by ethanol was significantly greater at A52S GlyR than WT when taurine was the agonist (Figure 4.6A). I saw no difference between A52S and WT when activated by glycine (Figure 4.6B), however, which contradicts earlier work that showed a decrease in glycine-evoked current enhancement in A52S relative to WT (Mascia et al., 1996).

It is possible that this discrepancy is due to differences in background levels of zinc in my experiments, compared to those of Mascia et al. (1996). Zinc contaminates reagents and buffers at low nanomolar concentrations, high enough to significantly enhance GlyR function, and is known to act synergistically with ethanol to further potentiate glycine-activated currents (Cornelison et al. 2014, 2017). It is therefore possible that Mascia and colleagues (1996) had greater concentrations of zinc contaminating their buffers. It is also possible that we used different isoforms of the α1 GlyR which may differ somewhat in function. The human α1 isoform 2 lacks an 8-

amino acid insertion in the intracellular domain that is present in isoform 1. All of my experiments were performed using isoform 2 but it is unclear which isoform was used by Mascia and colleagues.

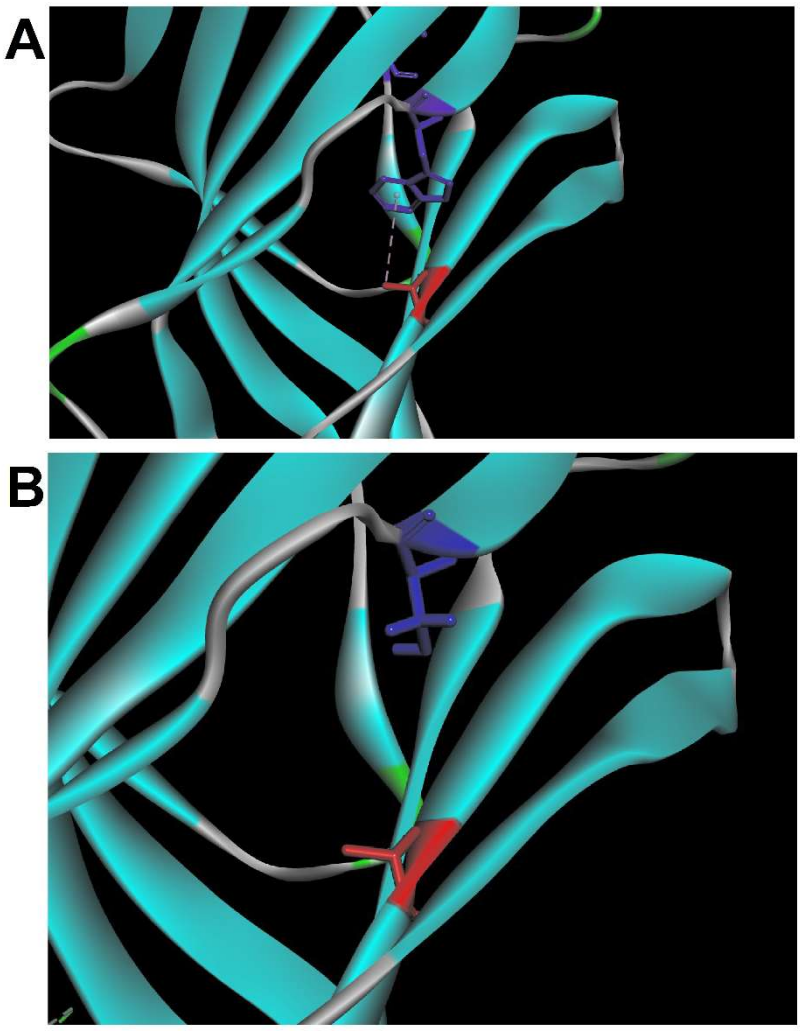


**Fig 4:12 - Structural model of the zebrafish α1 GlyR showing W170 and W170S mutation**

Model of a single subunit of the homopentameric glycine-bound α1 GlyR showing the intra-subunit interaction between (A) W170 in Loop F (shown in blue) and A212 (shown in red). (B) This interaction is disrupted by the W170S mutation (shown in blue).

Having observed a difference in ethanol modulation of low taurine currents at A52S α1 GlyR, I next compared currents produced by saturating concentrations of glycine and taurine and found that taurine produced peak current amplitudes that were ~20% of those seen with glycine (Figure 4.7A). This is significantly lower than WT indicating that the Po of taurine-bound receptors and therefore, the efficacy of taurine, is reduced by the A52S mutation. I next co-applied saturating taurine with 200mM ethanol. Consistent with my hypothesis that the magnitude of allosteric modulation varies inversely with agonist efficacy, the degree of current enhancement by ethanol was substantially higher in the A52S mutant (Figure 4.7B).

Zinc is a biphasic modulator of GlyR function that is found ubiquitously in the central nervous system and as a contaminant in our buffers (Frederickson et al, 2006a, 2006b; Hirzel et al, 2006; Cornelison and Mihic, 2014). Since zinc can act in a synergistic manner with other modulators to alter GlyR function, I was concerned that some of the changes in ethanol modulation I had seen were due, in part, to differences in zinc sensitivity between A52S and WT receptors. To rule this out, I measured zinc modulation of GlyR currents when activated by low ($EC_{4-10}$) and saturating (100 mM) concentrations of taurine. Co-application of taurine with potentiating (0.1 μM & 1 μM) and inhibiting (100 μM) concentrations of zinc revealed no significant differences in zinc sensitivity between A52S and WT (Figure 4.8), thus confirming that the increase in taurine-evoked current potentiation seen with A52S was ethanol-specific.

Recently, Cornelison et al., (2017) demonstrated the utility of the α1 W170S mutant, which is insensitive to enhancing concentrations of zinc, for assaying GlyR function without the confounding effects of zinc potentiation. Expanding on this work, I characterized the effects of taurine and modulation of taurine-evoked activity at W170S α1 GlyR. First, I constructed taurine concentration-response curves for W170S and WT GlyR, that showed no significant difference between the two receptor types (Figure 4.9), which is in agreement with the findings of Zhou et al. (2013). I then compared currents produced by saturating concentrations of taurine and glycine and found that the efficacy of taurine was comparable to glycine in the W170S mutant (Figure 4.10A). This provided the opportunity to further validate my earlier hypothesis regarding the relationship between agonist efficacy and the magnitude of allosteric modulation. If taurine is a high efficacy agonist, similar to glycine, at W170S then the Po of W170S GlyRs activated by high taurine concentrations should be close to 1 in the absence of any modulators. According to this model, I would therefore not expect to see any enhancement by ethanol of maximally-effective taurine currents, which is what I found (Figure 4.10B).

Cornelison et al. (2017) found that the W170S α1 GlyR displayed a significantly lower ethanol response than WT receptors when activated by $EC_{5-10}$ glycine in standard MBS buffer known to contain low nanomolar levels of contaminating zinc. This difference between receptors was eliminated when the zinc chelator tricine was added to the buffers. I performed similar experiments using $EC_{5-10}$ taurine and also saw greater

ethanol modulation of WT currents than W170S when background zinc was present (Figure 4.11A) but this was no longer the case when using MBS that contained 2.5mM tricine (Figure 4.11B).

Based to my hypothesis of an inverse relationship between the magnitude of allosteric modulation and agonist efficacy, one would have expected the enhancing effects of ethanol to be greater for WT than W170S receptors, even in the absence of zinc enhancement, if efficacy alone was being affected. Due to the large increase in taurine efficacy caused by this mutation, the degree of allosteric modulation would be reduced relative to WT where taurine has much lower efficacy. This was seen at saturating taurine concentrations (Fig. 4.10). The fact that W170S α1 GlyR still show ethanol enhancement at low concentrations of taurine (Fig. 4.11) also suggests that ethanol acts to left-shift taurine-concentration response curves, similar to its effects on WT receptors.

The zinc-insensitive phenotype of W170S has been attributed to a disruption of a high-affinity zinc binding-site (Zhou et al., 2013). This, however, does not explain the taurine-specific effects I observed. Upon examining the crystal structure of the zebrafish α1 glycine receptor from Du et al. (2015) in the glycine-bound state (3jae), I found that W170 is in close proximity to, and seems to interact with A212, a residue previously identified as being a key determinant in taurine activation of homomeric α1 GlyRs (Figure 4.12) (Schmieden et al. 1992). Based on the structural model, this interaction may be disrupted in W170S GlyR, which may facilitate the conformational changes necessary to transduce taurine binding into channel gating (Figure 4.12).

# Chapter 5: General Discussion, Conclusions and Future Directions

## 5.1 – Overview

Alcohol use disorders (AUDs) are one of the leading causes of preventable death, world-wide, and constitute a global economic burden. Some of the effects of ethanol may be attributed to its role as an allosteric modulator of glycine receptors (GlyRs) in the central nervous system. A better understanding how ethanol exerts its effects will provide insight into how alcohol use precipitates alcohol abuse and addiction and is crucial for the targeted development of therapies to combat addiction.

Determining how ethanol exerts its effects on the human body is a highly complicated problem, in part due to the large variety of different receptors, receptor subtypes as well as other proteins, such as adenylyl cyclase, that are affected by pharmacologically-relevant concentrations of ethanol. The fact that chronic ethanol use or withdrawal can induce changes in the subunit composition of these receptors adds another layer of complexity. Furthermore, these receptors respond to multiple agonists and numerous allosteric modulators, which may act synergistically. I sought to improve our overall understanding of how allosteric modulators influence receptor activity and provide insight into the mechanisms by which ethanol acts.

Zinc is ubiquitous in the body with tonic levels in the nanomolar range known to potentiate GlyR currents. It is also a common contaminant in our buffer at similar nanomolar concentrations. McCracken et al. (2010) showed that zinc acts synergistically with ethanol to enhance GlyR currents to a degree greater than either modulator would in

isolation. It is therefore important to consider the potential confounding effects of zinc when studying the effects of other allosteric modulators at these receptors.

The data presented in this dissertation provide evidence supporting a novel mechanism by which modulation of glycine receptors by zinc, ethanol and perhaps other compounds depends on the efficacy of the agonist activating the receptor. Furthermore, my research indicates that, in some cases, GlyR mutations that affect allosteric modulation do so by changing the efficacy of a particular agonist or agonists at the receptor. Consequently, further research regarding allosteric modulation of GlyRs and, potentially, related receptors, will need to also account for such changes.

### 5.2 – Allosteric Modulation of Wildtype Glycine Receptors

The glycine receptor is activated by glycine and taurine, endogenous compounds which may each be the primary GlyR agonist in specific regions of the central nervous system (Albrecht and Schousboe, 2005, Mori et al., 2002). Glycine is a high-efficacy agonist at the GlyR, with a Po of approximately 0.95 at saturating concentrations. In contrast, taurine has approximately 5% the efficacy of glycine and a Po of about 0.5 at maximally-effective taurine concentrations (Lape et al., 2008).

Previous work in our lab showed that ethanol, anesthetics, inhalants, and zinc significantly enhance GlyR currents elicited by saturating concentrations of the partial agonist taurine but have no significant effects at high glycine concentrations (Kirson et al., 2012, 2013). Biro et al. (2004) similarly observed a greater degree of allosteric

modulation of taurine- and β-alanine-evoked GlyR currents by propofol, ganisetron, and the neurosteroid analogs minaxolone and alphaxalone than at glycine-gated receptors. Still, we know relatively little about the nature of allosteric modulation of currents produced by low concentrations of taurine compared with glycine.

My initial goal was to characterize allosteric modulation of glycine receptors activated by low concentrations of taurine and see how it compared to modulation of similar currents gated by glycine. I used the two-electrode voltage clamp (2EVC) method on *Xenopus laevis* oocytes expressing wild-type homomeric α1 or heteromeric α2β GlyR to measure the degree of enhancement of glycine- versus taurine-activated GlyR currents, in the presence of either zinc (100 nM & 2.5 μM) or ethanol (50 mM and 200 mM).

Allosteric modulators exhibit the greatest effects at low agonist concentrations (Beckstead et al., 2000; Miller et al., 2005; Welsh et al., 2010). Based on prior experience, I expected to see greater potentiation of glycine currents because the concentration of glycine used fell much lower on its concentration response curve than the concentration of taurine needed to produce equivalent currents. Contrary to my initial expectations, however, I found that a similar degree of zinc enhancement of currents produced by $EC_{6.28}$ glycine and $EC_{22.69}$ taurine at α1 homomeric GlyR.

Upon further investigation, I saw concentration-dependent zinc potentiation of GlyR activity with greater potentiation, overall, of taurine than glycine currents (Figure 3.4) and negligible effects at high concentrations of glycine. The later was consistent

with previous data but the former was somewhat unexpected. I later observed a similar pattern of modulation by EtOH at α2β heteromeric and α1 homomeric GlyRs. This suggested that zinc and ethanol may be affecting GlyR currents via similar mechanisms and that allosteric modulators, in general, have greater activity at taurine-gated GlyRs.

We know from single-channel studies that modulators such as ethanol enhance glycine affinity by antagonizing unbinding from the receptor (Welsh et al., 2009). My data show that zinc and ethanol have very different effects when taurine is the agonist. Based on my own data and that of Kirson et al. (2012, 2013) it appears that zinc and ethanol increase the Po at glycine receptors activated by taurine, however, I observed a greater degree of zinc potentiation of taurine-evoked currents at lower EC values (Figure 3.4). Given this disparity in the degree of enhancement of currents produced by low and high concentrations of taurine, it seemed unlikely that only Po was being affected which led me to conclude that zinc was likely increasing taurine affinity at these lower EC values. This reaffirms the idea that the mechanisms by which zinc and ethanol enhance wildtype GlyR function differ between glycine and taurine.

### 5.3 – Role of Agonist Efficacy in Glycine Receptor Modulation

Based on my earlier observations summarized in Chapter 3, I hypothesized that the observed disparity in the magnitude of allosteric modulation at wildtype glycine receptors is due to differences in efficacy between glycine and taurine. There have been numerous studies comparing the effects of modulators, such as ethanol and zinc, on recombinant glycine receptors and GlyR mutants, but no one has studied whether

variations in alcohol sensitivity are in any way due to differences in agonist efficacy. This led me to wonder whether the results of our earlier experiments represent a generalizable mechanism for allosteric modulation of these receptors.

GlyR mutations can affect a number of receptor characteristics including affinity, Po and the response to allosteric modulators. Yet, no one has determined whether such differences in allosteric modulation result from mutation-induced changes in agonist efficacy. I hypothesized that, if a receptor is mutated such that a given agonist becomes more or less efficacious, there will be a corresponding change in the degree of allosteric modulation.

I tested this theory in Chapter 4 using mutant α1 GlyRs. I initially focused on mutations in the Loop 2 region as these affect both agonist and ethanol sensitivity (Mascia et al., 1996; Naito et al., 2014; Perkins et al. 2008, 2012). I examined a series of mutants, known as USERs, that were reported to have an enhanced sensitivity to low concentrations of ethanol when activated by glycine. I also generated a number of mutations in the α1 GlyR based off of the USER sequence, hoping to pinpoint the specific residues responsible for these changes. Despite the existence of several publications describing this ultra-low ethanol concentration sensitivity, I did not observe a similar effect in any of my mutants. Furthermore, I were unable to reproduce the USER data from the literature.

I next examined the effects of taurine at these USER mutants as this had not been previously investigated. Although USERs 1 and 3 did not differ from wildtype in

response to maximally effective concentrations of taurine, USER2 had a lower taurine response relative to glycine, and increased enhancement of taurine currents by 200 mM ethanol. Similarly, taurine had much lower efficacy relative to glycine at $\alpha1^{A52S}$ GlyRs and exhibited a greater degree of ethanol potentiation, as we predicted. I later determined that these changes were not due to a difference in zinc modulation at A52S, confirming that these effects were ethanol-specific.

Cornelison et al. (2017) recently validated the use of the $\alpha1^{W170S}$ mutant, which is insensitive to zinc potentiation, as a model for studying the effects of ethanol at GlyRs without the additional need to account for zinc contamination. In addition to its zinc insensitivity, W170S shows an enhanced taurine response. When I compared peak currents produced by maximally-effective concentrations of glycine and taurine, I found that taurine efficacy was comparable to that of glycine at W170S. I next examined the effects of 200 mM ethanol on saturating taurine W170S and WT GlyR. As expected, this drastic increase in taurine efficacy at W170S was accompanied by a significant reduction in ethanol potentiation which further supports my proposed mechanism.

### 5.4 – Future Directions

My work demonstrated that the nature of allosteric modulation of glycine receptors differs depending on the efficacy of the agonist gating the receptor. Based on my experiments with wildtype, USER2, A52S and W170S, I propose that there is an inverse relationship between agonist efficacy and allosteric modulation of glycine

receptors which should be addressed in any subsequent studies of glycine receptor modulation.

It is important to keep in mind that my measurements of taurine efficacy were all made relative to the activity of glycine. It could thus be argued that in some cases it wasn't necessarily taurine efficacy that went up, but instead that glycine efficacy went down. Single-channel recordings could distinguish between these two possibilities and should be performed in order to construct kinetic models that can be used to quantify agonist efficacies and open channel probabilities to further validate our model. Thus far, I have only explored modulation of glycine receptors. Additional experiments will need to be carried out to determine whether this mechanism is generalizable to other receptors.

It has yet to be determined how the W170S mutation causes such a dramatic change in taurine efficacy at these receptors. I proposed that disrupting intra-subunit interaction between W170 and A212 may be an important step in taurine-gating of the glycine receptor. Mutagenesis studies exploring the effects of different tertiary interactions between these regions of the protein would help confirm or disprove this hypothesis.

Structures of the GlyR in the active state are currently only available for glycine- and strychnine-bound receptors. The future availability of a taurine-bound GlyR structure would greatly enhance our understanding of the agonist-specific differences in conformation that follow glycine- vs. taurine-mediated activation of glycine receptors, and elucidate how these contribute to the functional disparities that are observed.

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